The Cellular RNA Helicase UAP56 Links Influenza A Virus Replication and the Antiviral Activity of Mx-Proteins

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To my parents, my brother and Karin

Summary

The type I interferon-induced Mx-proteins play a pivotal role in the first line of defence against viruses. Human MxA has been shown to be active against a large number of viruses including Influenza A virus, vesicular stomatitis virus, Bunyaviruses and Thogotovirus, but the exact molecular mechanism remains to be elucidated. There is evidence that Mx-proteins exert their antiviral activity via direct protein interactions. However, no direct interaction between Mx-proteins and any viral protein of Influenza A virus is known. Therefore we assessed whether cellular proteins, which are required for Influenza A virus replication represent a possible target of MxA. UAP56 and URH49 are two DExD/H box RNA helicases involved in mRNA export and spliceosome assembly, UAP56 was previously shown to stimulate influenza RNA synthesis *in vitro*. We were able to show a direct interaction between UAP56/URH49 and human MxA. Using a split GFP approach we demonstrated an interaction of UAP56/URH49 and MxA in the cytoplasm. Our results confirm the hypothesis of UAP56/URH49 shuttling between nucleus and cytoplasm. In addition UAP56 and URH49 are required for an efficient influenza A virus replication. Knockdown of UAP56 resulted in a 200-fold titer reduction for avian influenza A virus. Furthermore, we demonstrated that UAP56 is required during influenza A virus infection to prevent the formation of double stranded RNA, thereby preventing an activation of the type I interferon system. In summary we describe a novel interaction partner of MxA that is important for influenza A virus replication. In this study we propose a new mechanism how MxA exerts its antiviral activity by targeting cellular RNA helicases.

Zusammenfassung

Mx-Proteine sind Teil des Typ I Interferon-Systems und spielen eine wichtige Rolle bei der Bekämpfung zahlreicher Viren. Das menschliche MxA Protein hat antivirale Wirkung gegen eine Vielzahl von Viren, unter anderem gegen Influenza A Viren, Vesicular Stomatitis Viren, Bunyaviren und Thogotoviren. Der molekulare Mechanismus ist jedoch für die meisten Viren bisher nicht bekannt. Für Influenza A Viren konnte keine direkte Interaktion zwischen Mx-Proteinen und viralen Proteinen beschrieben werden. Unsere Fragestellung lautete, ob zelluläre Proteine, die für die Replikation von Influenza A Viren wichtig sind, ein mögliches Ziel von MxA sind. UAP56 und URH49 sind zwei DExD/H-box RNA Helikasen die für den Zusammenbau des Spliceosomes und mRNA Export verantwortlich sind. UAP56 stimuliert zudem die Influenza A Virus RNA Synthese *in vitro.* Wir konnten eine direkte Interaktion zwischen UAP56/URH49 und humanem MxA zeigen. Mittels eines Split-GFP Systems konnten wir zeigen, dass diese Interaktion im Zytoplasma stattfindet. Unsere Ergebnisse bestätigen die Hypothese, dass UAP56 und URH49 zwischen dem Nukleus und dem Zytoplasma “shuttlen” können. Zudem sind UAP56 und URH49 wichtig für eine effiziente Replikation von Influenza A Viren. Durch die Herunterregulierung von UAP56 konnten wir eine 200-fache Reduktion des Titers für aviäre Influenza A Viren zeigen. Außerdem konnten wir zeigen, dass UAP56 die Bildung von Doppelstrang-RNA während der Infektion von Influenz A Viren verhindert, welche das Typ I Interferon-System aktivieren würde. Zusammenfassend beschreiben wir hier einen neuen Interaktionspartner von MxA, welcher für die Replikation von Influenza A Viren wichtig ist. Wir schlagen in dieser Arbeit einen neuen Mechanismus für MxA vor, in welchem zelluläre RNA Helikasen und deren Interaktion mit MxA ein Teil des antiviralen Mechanismus sind.

Introduction

The influenza A virus

Influenza virus classification

Influenza A viruses (IAV) belong to the family of *Orthomyxoviridae* and can be divided into three different genera depending on the antigenic differences of their nucleo- and matrix proteins: type A, B and C. They also differ with respect to host range, variability of the surface glycoproteins, genome organization and morphology. Influenza A viruses are responsible for major pandemic and epidemic outbreaks throughout the past decades, as well as for the annual epidemic influenza outbreaks.

Influenza A viruses have the broadest host range of all three genera and are able to infect a large variety of mammals and birds. The main reservoir for Influenza A viruses are wild aquatic birds (**Figure 1**).

Influenza A viruses are further classified into subtypes based on the antigenicity of their haemagglutinin (HA) and neuraminidase (NA) surface proteins. So far 16 HA subtypes and 9 NA subtypes have been described [2]. The full nomenclature for each new isolate includes the type of virus, host of origin (except for human isolates), geographical site of isolation, strain number, and year of isolation. The antigenic serotype is given in parentheses, for example A/Puerto Rico/34/1908(H1N1). So far no subtypes have been described for Influenza B and C viruses.

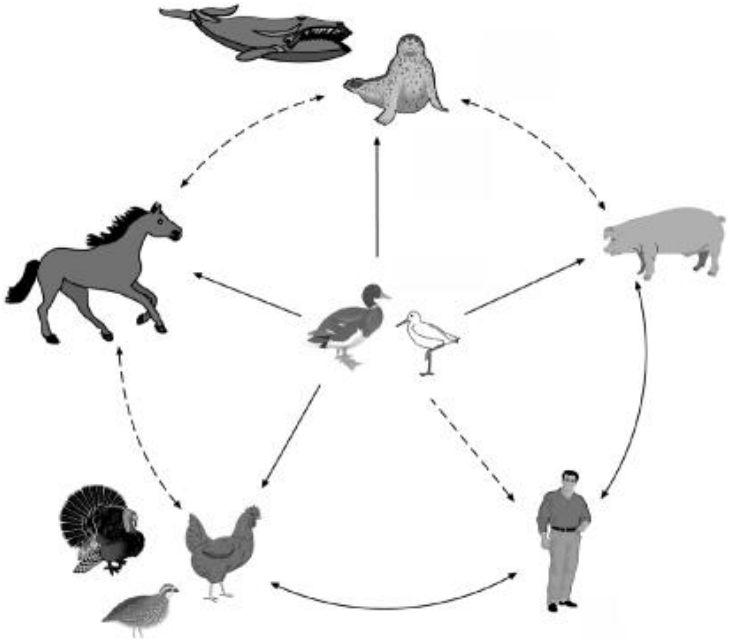

Figure 1. The reservoir of influenza A virus. The hypothesis is that wild aquatic birds are the primordial reservoir for all avian and mammalian influenza A viruses. Direct transmissions which have been demonstrated are shown with solid lines, whereas possible direct transmissions are shown with dotted lines.The five different host groups are based on phylogenetic analysis of the nucleoprotein genes of a large number of different influenza viruses [1].

Influenza A viruses are unique in their ability to cause recurrent seasonal epidemics of varying severity, as well as global pandemics which have been a major burden for humans in the last centuries. In the past centuries four major pandemics have been reported, the “Spanish Flu” (1918, H1N1), “Asian Flu” (1957, H2N2), “HongKong-Flu” (1968, H3N2) and the “Russian-Flu” (1977, H1N1). For the “Spanish-Flu” over 40 million deaths are estimated which accounts for more deaths than World War I [3]. The “Russian-Flu” is considered important nowadays, because it reintroduced the H1N1 as a circulating strain in the human population, today H1N1 and H3N2 strains are circulating in the human population.

Just recently in 2009 a new pandemic has spread over the globe, the so called “Swine-Flu” representing a novel H1N1 strain, which is a reassortant from avian, pig and human IAVs and caused a global pandemic in 2009/2010 [4].

Influenza A virus morphology and genome structure

Influenza A viruses are enveloped viruses whose genome consists of eight single stranded RNA segments of negative polarity, which code for at least 10 viral proteins [5]. The genome is about 13 kilo base pairs (kb) in size and the eight segments have a length ranging between 890 to 2341 bases. IAV particles are pleomorphic and have a size of 80-120 nm in diameter [5].

Each segment contains short 5` and 3` untranslated regions which have promoter activity and are partially complementary to form a so called “cork-screw” structure, in addition they are required for the recognition of the vRNA by the viral polymerase [6].

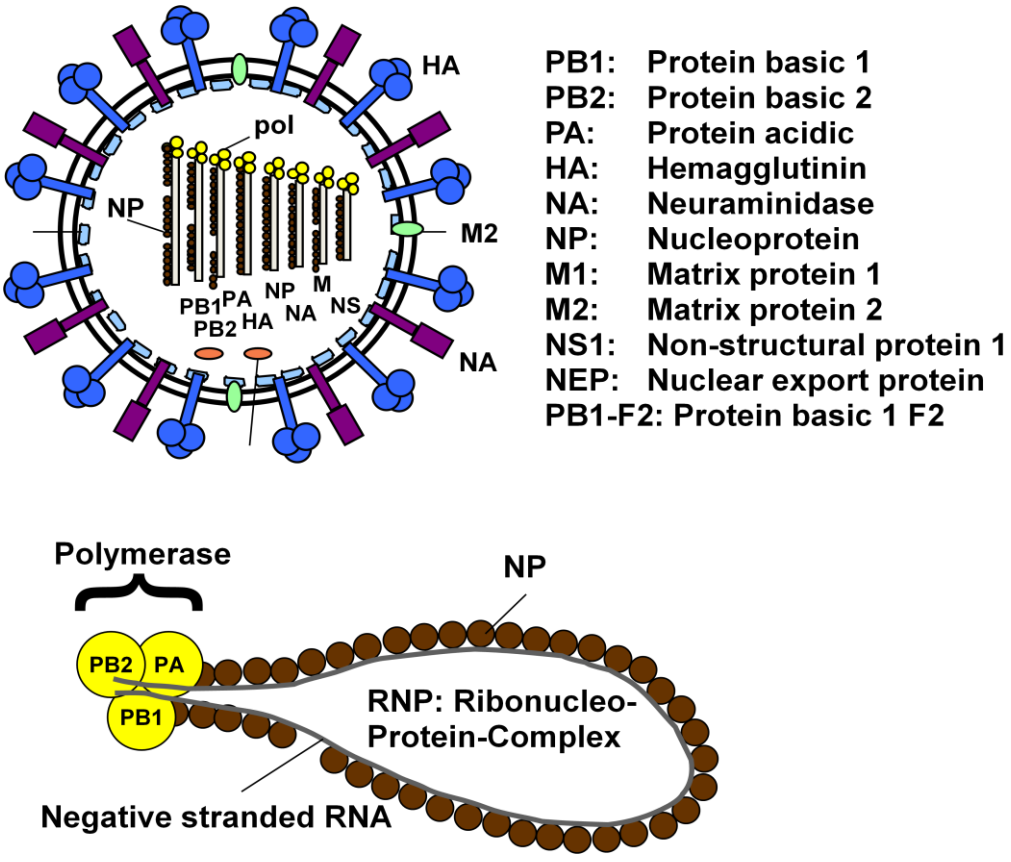

Figure 2: Schematic view of the influenza A virion and the ribonucleoproteincomplex (RNP). A schematic view of the virion is shown including all nine proteins in the virion, the NS1 protein cannot be found in the virion but only in infected cells. The negative stranded RNA is shown in a complex with the nucleoprotein and the polymerase subunits, also called the RNP. (Adapted from Jovan Pavlovic)

The lipid envelope of the virion is derived from the host cell membrane during viral budding from the cell surface and consists of a lipid bilayer. The viral glycoproteins HA and NA are encoded by the viral gene segments 4 and 6 and are embedded in the membrane of the virion. The HA forms a trimer which consists of three individual HA monomers. HA is synthesized as a single polypeptide chain (HA_0) and gets proteolytically cleaved into the active form, which consists of the two distinct subunits HA_1 and HA_2 connected by a disulfide bond [5, 7, 8]. Cleavage of HA_0 is essential for HA to mediate membrane fusion between the viral and the cellular membrane and represents a key step in the viral lifecycle [9]. In addition HA_1 is the major antigenic epitope of IAV against which host derived neutralizing antibodies are directed. The HA_2 forms the stem structure of the viral spike and contains a region of 20 amino acids at the N-terminus which is generally referred to as “fusion peptide” since it triggers the fusion between the viral and host cell membrane upon virus entry [9].

The NA is the second major surface antigen of IAV and is important for efficient viral budding. It displays enzymatic activity against terminal sialic acid residues linked to a sugar residue [5, 10]. NA is also of importance because it cleaves the sialic acids, which serve as the IAV cellular receptor, from the cell surface and therefore prevents a super-infection, as well as aggregation of newly formed virions on the cell surface.

Segment	vRNA(nt)	Protein	Amino acids	Functions
1	2341	PB2	759	subunit of RDRP; "Cap-snatching"
2	2341	PB1 PB1-F2	757 87	catalytic subunit of RDRP; elongation apoptosis
3	2233	PA	716	subunit of RDRP
4	1778	HA	566	haemagglutinin; surface-glycoprotein; receptor binding; membrane fusion
5	1565	NP	498	nucleoprotein; encapsidation of vRNA and cRNA; part of transcriptase complex; nuclear/cytoplasmic transport of vRNA
6	1413	NA	454	neuraminidase; surface-glycoprotein; receptor disruption, virus releasing
7	1027	M1 M2	252 97	matrix protein ion channel activity ; protecting HA-conformation
8	890	NS1 NEP/NS2	230 121	post-transcription regulator; inhibition of (i) pre-mRNA splicing, (ii) polyadenylation (iii) PKR-activation nuclear export factor

Figure 3: The Influenza A virus proteins and their functions. Adapted from [11]

The Matrix protein 2 (M2) is the third integral membrane protein, and consists of a homotetramer. M2 is a pH-activated proton channel and is essential for uncoating in the viral life cycle. It mediates proton uptake from the acidified endosome into the viral particle and thereby disrupts the interaction between the viral genome and the matrix protein 1 (M1) [5, 12, 13].

The viral RNA is assembled with the RNA-dependent RNA polymerase (RDRP), which is a complex of PB1, PB2 and PA which form the viral ribonucleoproteincomplex together with the nucleoprotein (vRNP) [5] (**Figure 2**).

As the main component of the RNP the influenza NP encapsidates the viral genome but in addition, also plays a major role in transcription of the RNA, replication and packaging [14]. The IAV NP is associated with the viral RNA, serving as a scaffold and also requires M1 to form helical structures [15]. It has been shown that free NP is needed for vRNA synthesis, which was believed to happen late in viral infection. Recently it was shown that vRNA synthesis occurs throughout the whole time of infection but only seems to be stable if enough RDRP complexes and NP are present to protect the newly synthesized vRNA

[16-18]. Upon export of the viral RNPs via the CRM-1 export pathway, NP remains tightly associated with the vRNA, PB1 and PB2 [14]. A layer of M1 protein surrounds the viral RNP. M1 is the most abundant protein of IAV and is thought to serve as an adaptor between the lipid envelope and the viral RNPs. In addition M1 is a maturation marker in the IAV replication, since association of M1 to the RNPs is important for RNP export later in the infection cycle [10, 13, 19-21].

Like segment 7, segment 8 also encodes for two proteins, the non-structural protein 1 (NS1) and 2 (NS2/NEP), NS2 mRNA is spliced from the primary mRNA, whereas the unspliced mRNA codes for NS1 (**Figure 3**).

The NS1 protein is not found in the virion and is only present in infected cells. NS1 was shown to be a viral antagonist of the IFN system (IFNα/β), since a NS1 deficient strain can only replicate in cells, which lack crucial parts of the IFN system [22, 23]. Furthermore, NS1 has been shown to have ssRNA and dsRNA binding activity, therefore it is able to sequester viral dsRNA and downregulate the activation of the dsRNA activated protein kinase R (PKR), NF-κB and IFN regulatory factor 3 (IRF-3) [24-26]. Recently, IAV NS1 was described to inhibit the cellular RNA helicase retinoic acid-inducible gene I (RIG-I), which is a cytoplasmic sensor for ssRNA bearing 5`-phosphates [27, 28]. RIG-I forms stable complexes with RNA containing a 5`-phosphate and during infection NS1 is recruited to such complexes via an RNA binding domain, thereby preventing a sensing of the viral RNA [27]. Additional studies have also shown an anti-apoptotic effect of NS1 [29].

In contrast to NS1, the NS2 protein is present in low copies in the virion. NS2 is nowadays also called nuclear export protein (NEP) since it is important for the export of newly synthesized RNPs from the nucleus into the cytoplasm [30].

Replication of influenza A virus

Upon infection IAV binds to sialic acid residues on the cell surface which are ubiquitously present on most cells. IAV mainly infects cells in the upper and lower respiratory tract, but can also infect macrophages or dendritic cells [10, 31].

Different IAV HAs show receptor specificity for varying glycosidic linkages between the terminal sialic acid and the following galactose residue [9]. Human IAVs primarily bind to an α2-6 attached sialic acid, whereas avian IAVs bind to an α2-3 attached sialic acid [32].

The binding of the virion to the cell surface induces a receptor-mediated endocytotic uptake of the virion, during which the virus particles are internalized by the host cell plasma membrane. Subsequent fusion of the vesicles with endosomal particles leads from mild acidic early endosomal compartments to late more acidic compartments. A crucial step in the IAV life cycle is the fusion of the viral and the endosomal membrane at a pH of about 5-6. Upon pH decrease the HA undergoes major conformational changes, and the fusion peptide of HA_2 is exposed which leads to an insertion into the endosomal membrane followed by fusion of the two membranes [8, 9] (**Figure 4**)**.**

The M2 protein serves as an ion channel and is important for the pH dependent uncoating (loosens the interaction between NP and M1) and release of the viral RNPs into the cytoplasm of the cell and further transport via the nuclear pore complex (NPC) to the nucleus. This transport is mediated via a nuclear localisation signal (NLS) in the NP [14, 33] (**Figure 4**).

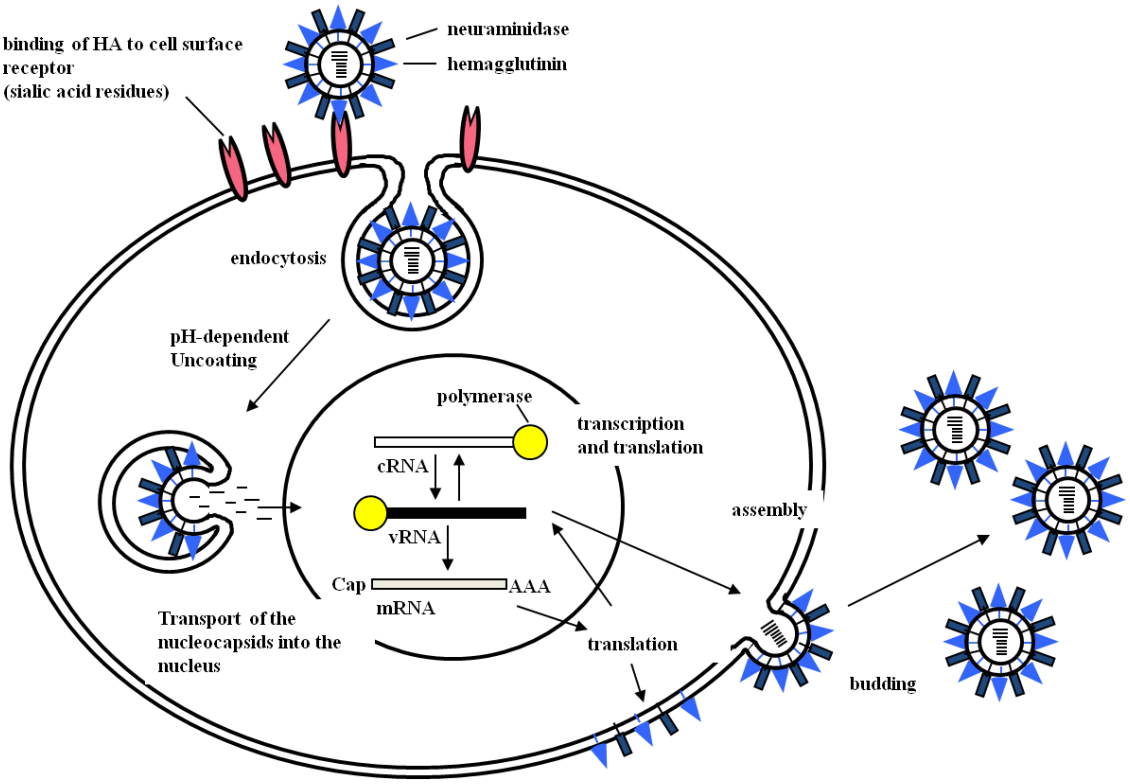

Figure 4: Influenza A virus replication cycle. Schematic view of the steps in the IAV replication cycle.

Once the RNPs are transported into the host-cell nucleus the associated polymerase complex begins with the primary transcription of mRNA, which also requires the cellular RNA polymerase II [34]. The viral RDRP cannot initiate IAV mRNA synthesis since m7GpppXm-containing capped primers (10–13nt long, containing 5`-GCA-3` at their 3` proximal ends) are needed. This process involves “stealing” of the 5`cap sequence from cellular mRNAs and is commonly referred to as “cap-snatching”. Hereby, the endonuclease activity resides in the PA subunit of the polymerase, whereas PB1 is required for elongation of the nascent mRNA [35]. In addition to the production of viral mRNAs cap-snatching also induces a “host-cell shutoff” in favoring synthesis of viral proteins over cellular ones [10]. Transcription of viral mRNA continues until it reaches the polyadenylation site located 15-22nt from the 5`end of the vRNA.

The vRNA (minus strand) does not only serve as a template for mRNA it is also a template for the RDRP to produce viral cRNA (plus strand), which in turn leads to an increased synthesis of vRNA.

In contrast to mRNA synthesis vRNA and cRNA can be transcribed in a primer-independent manner leading to 5` triphosphorylated ends. Additionally, the poly-A signal is ignored in cRNA synthesis [5, 14]. Export of viral mRNAs occurs via different export pathways, involving NFX1 and several additional factors [36].

After their translation PB1, PB2, PA and NP are transported back to the nucleus, whereas HA, NA and M2 are translated into the lumen of the endoplasmic reticulum (ER) and undergo an oligomerization before being transported to the golgi and subsequently to the plasma membrane [5, 10, 37]. While being transported through the ER and the golgi, HA and NA are heavily glycosylated and further processed.

In contrast to the viral glycoproteins newly synthesized PB1, PB2, PA and NP are transported to the nucleus where they associate with newly synthesized vRNA to form viral RNPs. Nuclear export of the vRNPs is mediated by NS2 and is CRM-1 dependent [30]. After being transported to the cytoplasm, vRNPs associate with viral membrane proteins and budding of the virions occurs at the plasma membrane of the infected cell [20, 30, 38].

Antigenic variation of influenza A virus

Influenza viruses are an ongoing risk for the human population, because of their ability to exchange genome segments and a high error rate of the RDRP. In combination those two mechanisms increase the diversity of the IAV antigenicity.

Point mutations in the IAV genome are commonly referred to as “Antigenic drift”and mainly occur in the HA and NA glycoproteins. This allows the virus to adapt to selective pressure given by circulating neutralizing antibodies [39, 40]. Virus strains resulting from “antigenic drift” usually result in small epidemics, since partial immunity from circulating cross-neutralizing antibodies exists.

Highly virulent strains, which cause major pandemics, occur via a mechanism called “antigenic shift”. “Antigenic shift” is the change genomic segments to form a new subtype of influenza A virus. One ore more viral segments can be exchanged between different virus strains and the new virus is therefore immunologically distinct from previously the circulating isolates [10]. Most commonly pandemic strains are a mixture of both avian and human IAVs, but the recent pandemic in 2009 (H1N1) was a variant containing human and avian segments as well as segments with swine origin, which proofs the theory of swine being a “mixing vessel” for IAV [41].

Currently only H1N1 and H3N2 strains are circulating in the human population. The 2009 pandemic H1N1 strain appears to have replaced the former circulating H1N1 strain.

DEAD-box RNA helicases

The DEAD box family of proteins includes a large number of proteins which play important roles in the RNA metabolism. Different members of this family have been shown to act as RNA helicases which are involved in unwinding RNA structures, dissociation of RNA-protein complexes in cellular processes, transcription, splicing, RNA export and translation [42].

DEAD box helicases belong to a large superfamily (SF2) of proteins which is conserved throughout bacteria, viruses and humans. All proteins contain seven or eight conserved motifs whose characteristics are used to subgroup members into individual families [43]. The name of the family is dervived from the amino-acid sequence D-E-A-D (Asp-Glu-Ala-Asp) from a specific motif. Besides DEAD-box helicases very closely related families like DEAH, DExH and DExD-box helicases exist, which are commonly referred to as DExD/H-box helicases [43, 44] (**Figure 5**).

Since the early 1980s over 500 DEAD-box proteins and RNA helicases from the DEAD-box related families have been described [45].

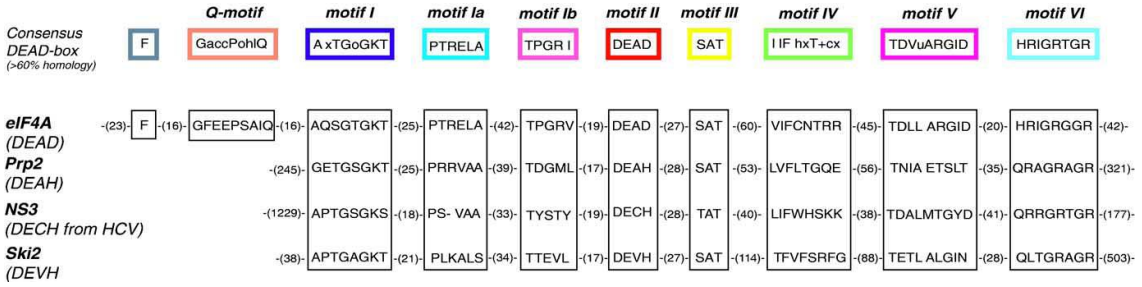

Figure 5: Conserved motfis in the DEAD-box and related DExD/H-box families. Sequences of the conserved motfis from the *S. Cerevisiae* eIF4A (DEAD-box protein), Prp2 (DEAH-box protein), NS3 (DECH helicase from hepatitis C virus) and Ski2 (DExH, Ski2 family). From [46]

Despite the ubiquitous function of DEAD-box proteins in processes involving RNA many precise roles or functions still remain unclear and have to be further elucidated. As a subgroup of DEAD-box helicases, DExD/H-box helicases can fulfill almost all functions involving RNA activities. Since the helicases UAP56 and URH49 belong to the DExD/H-box helicases a short introduction on this particular subgroup will be given.

UAP56 and URH49

UAP56 has been first described in 1997 as U2AF65-associated protein of 56 kDa required for U2 snRNP addition to the branch point region during pre-spliceosome formation *in vitro* [47]. In addition to UAP56 a close paralogue, which is 90% identical to UAP56 has been described and termed URH49 (UAP56-related helicase, 49 kDa, also called DDX39) [48] **(Figure 6**).

Both DExD/H-box helicases are involved in mRNA splicing and nuclear export of mRNAs [49] and have been described to be essential in eukaryotes. In lower eukaryotes like *S. Cerevisiae* or *D.melanogaster* only one protein corresponding to UAP56 is expressed, whereas in mice and humans UAP56 and URH49 are expressed. Interestingly, birds only express UAP56 and lack the close paralogue, a fact that will be further addressed in the discussion section of this work.

Although both proteins are 90% identical and exhibit similar functions they are encoded on different chromosomes in the human genome (UAP56 on chromosome 6 and URH49 on chromosome 19). Functional studies of UAP56 and URH49 show the ability of both to complement the otherwise lethal deletion of Sub2, the yeast homologue [48]. In addition both proteins have been shown to interact with the cellular mRNA export factor ALY/REF, recruiting it to spliced mRNA-protein complexes, which is needed for export of mRNA [50]. Since most of the studies regarding the function of DExD/H-box helicases have been performed with UAP56 but not URH49, we will mainly focus on the function of UAP56 in this work.

UAP56 is one of the few DExD/H-box proteins whose crystal structure has been solved for the whole protein, which gives a closer insight into possible mechanisms and requirements of the helicase in cellular processes [51, 52]. Further biochemical characterization of UAP56 revealed an involvement in spliceosome assembly and mRNA export. UAP56 was shown to be an RNA-stimulated ATPase as well as an RNA helicase which can unwind dsRNA substrates with 5` or 3` overhangs and blunt ends *in vitro* [53].

During transcription of a gene, splicing of the nascent mRNA (pre-mRNA) is a critical event and is carried out by the spliceosome complex. Intron containing pre-mRNAs have to be spliced to remove the introns before the mRNA can be transported out of the nucleus for translation [54]. During this process UAP56 recruits the ALY/REF adaptor protein to the mRNA prior to nuclear export [50, 55]. The heterodimeric export factor TAP/NFX1 binds to ALY/REF, displacing UAP56 from the complex, leading to export of the mature mRNA through the nuclear pore complex [56].

UAP56 could also be shown to be important for the export of mRNAs derived from either intronless or intron containing transcripts, which makes UAP56 not exclusively coupled to export of spliced mRNAs [50, 57].

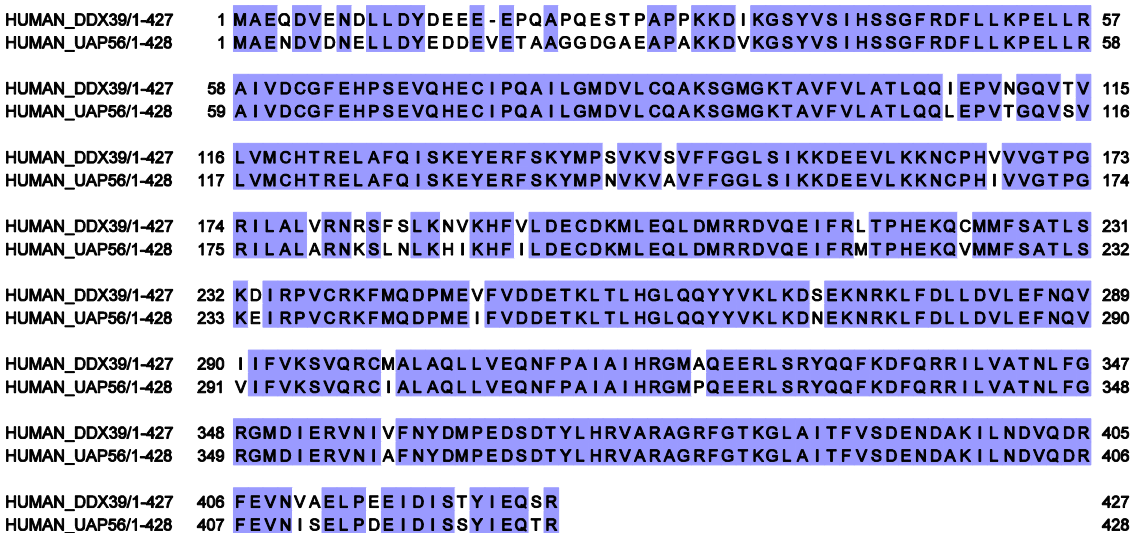

Figure 6: Sequence identity of UAP56 and URH49 (termed DDX39). Sequence identity between the human DExD/H proteins UAP56 and URH49 is shown in blue.

Besides important cellular functions UAP56 was also described to interact with N-terminus of IAV NP and enhance viral RNA synthesis [58]. So far nothing is known about URH49 and a possible interaction with IAV NPs or an enhancing effect on viral RNA synthesis.

Recently Read and Digard showed that the viral M1, M2 and NS1 mRNAs are exported via an UAP56/NXF1 dependent pathway, whereas segments 1 and 5 (PB2 and NP) seemed to be less dependent on UAP56 [36]. Export of unspliced and spliced mRNAs is dependent on UAP56, whereas intronless mRNAs might use additional export pathways [36]. Concerning export of IAV mRNAs nothing is known about URH49. These findings about export of viral mRNAs can be combined with different suggestions that IAV needs to recruit several cellular factors for an efficient viral replication. UAP56 and URH49 have also been described to be involved in the export of mRNAs and replication of human cytomegalovirus (HCMV) [59]. Both helicases were shown to be necessary for mRNA export activity in combination with the viral pUL69 protein, which further contributes to efficient viral replication [60]. For HCMV a direct binding of pUL69 to UAP56 or URH49 was observed. PUL69 is required for efficient export of

unspliced HCMV mRNA into the cytoplasm [59, 61]. A similar mechanism could also be possible for IAV. Until now only a small effect on viral replication could be shown for UAP56 and the mechanism for this remains poorly understood. For recent studies only human IAV strains where used and in addition nothing is known about the role of URH49 in IAV replication.

Myxovirus resistance proteins (Mx)

The interferon (IFN) type I system plays a pivotal role in the first line of defense against viral infections. Interferon synthesis and secretion is rapidly induced in infected cells due to the activities of virus sensors such as the toll like receptors 3 or 7 or DEAD-box helicases RIG-I and MDA5 [27, 28]. These proteins recognize and bind virus-derived single stranded or double stranded RNA and induce a signalling cascade resulting in the activation of IFN type I genes [62]. IFN is secreted and binds to its cognate receptor predominantly in a paracrine fashion on neighboring cells, thereby inducing the synthesis of approximately 300 IFN type I-induced proteins. The combined activities of these proteins result in the generation of a so called antiviral state restricting the replication of many viruses [63]. Myxovirus resistance proteins have been first described in 1962 by J. Lindenmann to confer resistance to IAV in mice [64]. Until today Mx-proteins have been described in a number of species but we will focus on the murine Mx1 protein and the human MxA protein in this work. Mx-proteins are exclusively induced by IFN α/β and human MxA exerts a broad antiviral activity against orthomyxoviruses, paramyxoviruses, bunyaviruses, rhabdoviruses, togaviruses, and hepatitis B virus [65].

Mx-proteins belong to a functionally diverse family of large GTPases with significant homologies to mammalian dynamins and yeast VPS-1 (vacuole protein sorting) [65]. Human MxA is homologous to dynamin sharing an N-terminal G-domain (GTP binding and hydrolysis), a less well-defined middle

domain and a GTPase effector domain at the C-terminus. In humans two distinct forms are encoded on chromosome 21 that are termed MxA and MxB, of which only MxA has been shown to have antiviral activity. It was shown that mice which constitutively express MxA can survive otherwise lethal infections with IAV even if they lack the interferon receptor (IFNAR-/-) and therefore are not able to mount an antiviral state, which indicates that MxA is a key component of the interferon system [66].

Molecular properties of human MxA and mouse Mx1

MxA is localized in the cytoplasm and shows a characteristic granular staining pattern. The observed granules most likely represent Mx assemblies, which are associated with the smooth endoplasmic reticulum (ER) [67, 68]. All Mx-proteins share the common G-domain and a GTPase effector domain, but differ in length and size. In contrast to human MxA, murine Mx1 is located in the nucleus and shows a stronger inhibition of IAV replication compared to MxA. Mx-proteins are organized in an N-terminal moiety that comprises the GTP-binding domain and a C-terminal moiety that serves as the antiviral effector domain (**Figure 2**). Mx-proteins exhibit an intrinsic GTPase activity that is essential for their antiviral function, nevertheless the exact function of the GTPase activity remains unclear and has to be further elucidated [69].

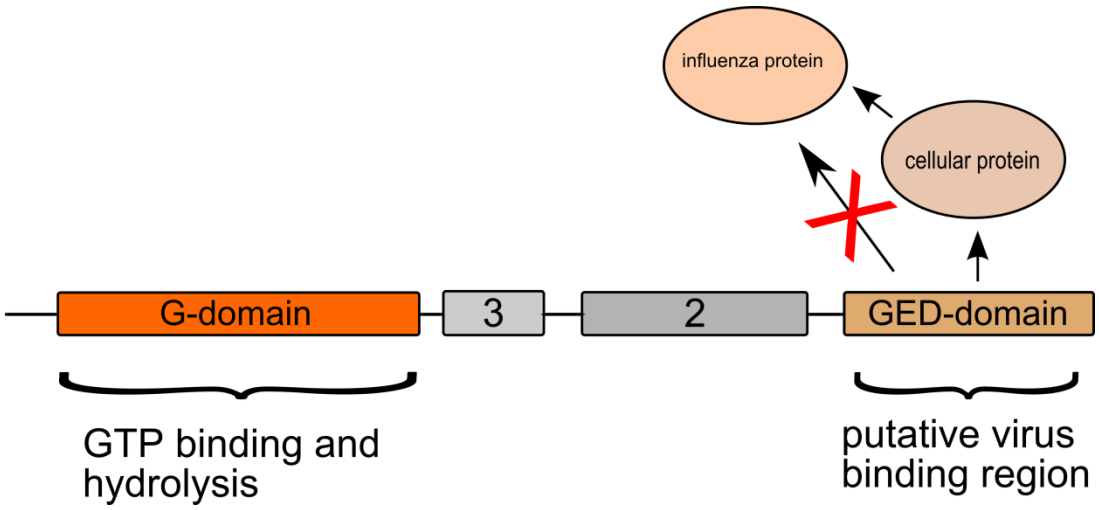

Figure 7: Schematic drawing of human MxA. The N-terminal GTP-binding domain is shown in orange, middle domain is divided into two domains termed 2 and 3 since the biological functions of this domain are poorly understood, 3 and 2 are also referred to as the CID (central interactive domain). The C-terminal GED-domain is shown in light brown; arrows indicate a possible function of the GED-domain. No direct interaction between MxA and any IAV protein could be shown, but a cellular linker protein could be the missing link for the antiviral mechanism of MxA.

Recently the crystal structure of the stalk region of MxA has been solved. The crystal structure further supports previous findings about oligomerization and GTP hydrolysis to have functional implications not only for MxA but also to all members of the dynamin family [70]. The stalk of MxA was shown to form a four-helical bundle, resulting in a structure that is important for the highly oligomeric order of MxA. Several mutants that were not able to form the four-helical bundle were lacking antiviral activity against IAV, indicating an important function for the antiviral activity of MxA [70]. Though the G-domain and the GTPase effector domain are known to be important for the antiviral activity, the molecular mechanism how MxA or Mx1 inhibit the IAV replication is not known. For thogotoviruses, another member of the *Orthomyxoviridae*, an interaction with the nucleocapsid was shown that inhibits the nuclear import of the viral nucleocapsids [71]. The replication of La Crosse virus (LACV), a member of the *Bunyaviridae,* is also inhibited by MxA via a sequestration of the nucleocapsids protein into perinuclear complexes [71-74]. No such mechanism could be demonstrated for

IAV or any other virus inhibited by MxA. However, there is evidence at which step MxA or Mx1 could inhibit IAV replication. The nuclear localized murine Mx1 was shown to inhibit viral mRNA synthesis, in contrast the cytoplasmic human MxA only blocked the amplification of the viral genome without affecting primary transcription [75]. These two different targets might be a result of the different intracellular localization of both proteins, since MxA was not shown to be present in the nucleus of infected cells. Interestingly, an MxA variant with the NLS of the simian virus 40 (SV-40) large T-antigen attached to its N-terminus, was shown to have a nuclear localization and inhibit IAV replication at the level of primary transcription, showing the importance of the cellular localization in terms of the antiviral activity [76]. Since no direct interaction between MxA and any IAV protein was detected, it might be an indirect interaction via a mediating cellular protein that is important for the antiviral effect. For MxA very few interactions with cellular proteins are known. MxA was shown to interact with actin and is believed to have a membrane binding activity [77]. For Mx1 a small number of interactions with the SUMO-1 protein modification system has been described, but so far none of this interactions proofed to be important for antiviral activity [77, 78].

Aims

No direct interaction with any IAV protein was shown for MxA so far, which makes it possible for a cellular protein to mediate between MxA and components of the virus. In 2001 the cellular helicase UAP56 was shown to interact with the nucleoprotein of IAV and the interaction was verified in 2007 [58, 79]. UAP56 is a cellular RNA helicase involved in mRNA export and spliceosome assembly [51, 80, 81]. Recently a close paralogue of UAP56 termed URH49 has been described having 90% identity on the amino acid level [48]. We addressed the question whether both RNA helicases interact with IAV NPs and play a role in IAV replication. Additionally we analyzed whether UAP56 and/or URH49 were able to bind MxA and/or Mx1 in different binding assays, making this a possible conjunction between the IAV NP or vRNP and MxA/Mx1. The goal of this study was to further elucidate the molecular mechanism of the interferon-induced human MxA and how it confers resistance to IAV and to show an involvement of cellular factors in the antiviral activity of MxA.

Manuscript 1

The cellular RNA helicase UAP56 is required for prevention of dsRNA formation during influenza A virus infection

The cellular RNA helicase UAP56 is required for prevention of dsRNA formation during influenza A virus infection

Running title: **UAP56 prevents influenza virus dsRNA formation**

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Contributions: C.W. and J.P designed the experiments. C.W performed all experiments, ALPHA screens were performed by C.W and D.M. Titration experiments were performed by C.W. and T.L. with the help of E.M.

Abstract

The cellular DEAD box RNA helicase UAP56 plays a pivotal role for efficient transcription/replication of influenza A virus. UAP56 is recruited by the nucleoprotein (NP) of influenza A viruses and recent data revealed that the RNA helicase is required for the nuclear export of a subset of spliced and unspliced viral mRNAs. The fact that influenza viruses do not produce detectable amounts of dsRNA intermediates during transcription/replication suggests the involvement of cellular RNA helicases. Hence, we examined whether the RNA unwinding activity of UAP56 or its paralogue URH49 plays a role in preventing the accumulation of dsRNA during infection. First, our data showed that not only UAP56 but also its paralogue URH49 can interact with NP of avian and human influenza A viruses. SiRNA-mediated depletion of either RNA helicase efficiently reduced transport of M1 RNA as well as NP mRNAs into the cytoplasm. Moreover, we found that virus infection of UAP56-depleted cells lead to a rapid accumulation of dsRNA in the perinuclear region. In parallel, we observed a robust virus-mediated activation of dsRNA-dependent protein kinase R (PKR) indicating that the cellular RNA helicase UAP56 may be recruited by the influenza virus to prevent dsRNA formation. Accumulation of dsRNA was blocked when actinomycin D or cycloheximide was used to inhibit viral transcription/replication or translation, respectively. In summary, we could demonstrate that UAP56 is utilized by influenza A viruses to prevent the formation of dsRNA and hence the activation of the innate immune response.

Introduction

Influenza A viruses cause a highly contagious respiratory disease in humans and have been responsible for periodic wide-spread epidemics, or pandemics that have caused high mortality rates (22). The most devastating pandemic occurred in 1918, resulting in 20 to 50 million deaths worldwide. Influenza viruses are enveloped and their genome consists of eight negative stranded RNA segments coding for at least 10 proteins. While the majority of their viral mRNAs are intronless (mRNAs of segments 1 to 6), segments 7 (M) and 8 (NS) generate spliced as well as unspliced mRNAs (16). The viral genome is transcribed and replicated within the host cell nucleus by the virus encoded RNA-dependent RNA polymerase complex consisting of the three subunits PB1 (protein basic 1), PB2 and PA (protein acidic). Synthesis of viral mRNA involves a “cap snatching” mechanism, whereby the cap structures of cellular precursor mRNA is cleaved 12 to 15 nucleotides downstream of the cap by the endonuclease activity of PA (6). The capped oligonucleotides serve as primers for the initiation of the viral mRNA synthesis. Replication of the viral genomic RNA involves synthesis of a positive stranded complementary intermediate (cRNA) of the genomic RNA. Since the genome of influenza viruses does not encode for a helicase, which is required for the unwinding of dsRNA replicative intermediates, it has been suggested that the influenza virus polymerase complex may recruit cellular RNA helicases. In support of this, Momose and colleagues demonstrated that the nucleoprotein (NP) of influenza A virus binds to the DEAD box helicase UAP56 and that interaction between UAP56 leads to increased vRNA synthesis *in vitro* (21).

UAP56 plays a pivotal role in spliceosome assembly as well as export of spliced and unspliced mRNA from the nucleus into the cytoplasm. UAP56 was first identified as an essential splicing factor that is required for pre-spliceosome and mature spliceosome assembly (9, 33, 34). Hereby, UAP56 as part of the transcription-export complex (TREX) recruits the Aly/REF nuclear export adaptor protein to the exon-junction complex (EJC) bound to pre-mRNAs and delivers it to

the export receptor NXF1 (4, 31). Further, UAP56 appears to be involved in proper translocation of mRNAs in the cytoplasm (20). URH49/DDX39 is a paralogue of UAP56 exhibiting 90% amino acid identity. URH49 appears to have a very similar function to UAP56 in nuclear RNA export (15, 27). Like UAP56, URH49 is able to interact with the nuclear export adaptor protein Aly and can partially complement the function of Sub2p the yeast homologue of UAP56 (27). Both URH49 and UAP56 can be detected in all mammalian cells and tissues but exhibit different expression profiles. While URH49 expression is clearly increased in proliferating cells, UAP56 levels remain constant (27). Concomitant downregulation with URH49 and UAP56 specific siRNAs leads to retention of poly(A)+ RNA in the nucleus and to cell death within 72 hours (15). So far there is no data available that would link URH49 to spliceosome assembly or cytoplasmic targeting of mRNPs. Growing evidence suggests that influenza viruses exploit the primary cellular mRNA nuclear export pathway for directing their mRNAs to the cytoplasm (2, 28, 38). Subsets of spliced and unspliced influenza virus mRNAs are exported into the cytoplasm in an UAP56/NFX1-dependent fashion. Read and colleagues recently showed that UAP56 is involved in the nuclear export of M1, M2 and NS1 mRNA, while the export of other viral mRNAs such as those encoding NS2, NP and HA appear to be independent from UAP56 (28). Hence, UAP56 participates in the nuclear export of spliced as well as unspliced viral mRNAs. In contrast the nuclear export of vRNPs is mediated by the CRM-1 pathway independent of UAP56 (8).

In this study, we first sought to determine whether UAP56 and URH49 play a role in replication/transcription of influenza virus RNA. We observed that both URH49 and UAP56 interact with NP protein from both avian and human influenza virus strains. Using siRNAs specific for either URH49 or UAP56 we found that downregulation of UAP56 or URH49 reduced accumulation of NP mRNAs in the cytoplasm. Most interestingly, we found that virus infection of cells with downregulated UAP56 leads to the rapid accumulation of dsRNA in the perinuclear region. In parallel, we observed a robust virus-dependent activation of dsRNA-dependent protein kinase R (PKR) in these cells, indicating that the function of the cellular RNA helicase UAP56 may be redirected by the influenza virus to evade the antiviral function of the interferon type I system.

Material and methods

Cells and viruses: A549 and HEK 293T (ATCC) cells were cultured in DMEM (Gibco) supplemented with 10% FCS, 1% Pen/Strep (Gibco) and 1% Glutamax (Gibco). The human influenza strain A/PR8/34/(H1N1) (PR8), the avian strain A/FPV/Bratislawa/79/(H7N7), and the vesicular stomatitis virus Indiana (VSV) were previously described (26). Infections were carried out in DMEM 2% FCS, 1% Pen/Strep and 1% Glutamax for FPV and VSV. For PR8 DMEM with 0.2% BSA (Roth), 1% Pen/Strep and 1% Glutamax and 0.5µg/ml trypsin was used. Cells were washed once with infectious medium afterwards infections were done for 30 minutes at room temperature, virus was aspirated and the cells were incubated in infectious media for the indicated amounts of time. Viral titers were determined by $TCID_{50}$ with supernatants from infected cells. Madine darby canine kidney cells (MDCK) were used for virus titrations. Titrations were performed as previously described (26). Statistical significance was determined using the paired t test.

Transfections and siRNA knockdowns: 293T cells were transfected at 80% confluency with jetPEI transfection reagent (Polyplus transfection) according to the manual. Validated stealth siRNAs were purchased from Invitrogen, medium GC control siRNA (Invitrogen, 12935-300), validated pooled siRNAs were used against URH49 (DDX39HSS141113, DDX39HSS141114, DDX39HSS141115, NM_005804.2) and UAP56 (BAT1HSS111847, BAT1HSS111848, BAT1HSS111 849, NM_004640.5). SiRNA transfections were done using HiPerFect (Qiagen) as described in the manufacturers manual. Transfections were performed in suspension using $6x10^4$ cells per 24-well and 30nM siRNA. Knockdowns were confirmed via western blot analysis and qPCR.

Western blot and co-immunoprecipitation assays: 293T cells were grown in 10cm cell culture dishes (TPP) and transfected at 80% confluency with the indicated amounts of plasmid. 48 hours after transfection cells were lysed in

500µl lysis buffer (0.5 % Triton X-100, 20mM TRIS pH 7.5, 100mM NaCL, 50mM β-glycerolphosphate, 50mM sodiumfluoride, 1mM sodiumorthovanadate and a protease inhibitor cocktail (Roche). Coimmunoprecipitations were perfomed with 1µg mouse anti-FLAG antibodies (SIGMA) for 4 hours at 4°C.
Immunoprecipitations were done at room temperature for 1h using 50 µl protein-G beads (Dynalbeads, Invitrogen). Samples were washed three times with lysis buffer and beads were taken up in 40µl SDS-Laemmli buffer and heated for 5 minutes at 95°C. Samples were loaded on 10% SDS-gels followed by immunoblot analysis with different antibodies. Anti-FLAG antibody (1:3000, SIGMA) against mouse and rabbit, anti-URH49 (1:750, Acris), anti-UAP56 (serum from mice immunized with a peptide of human UAP56), anti-NP mouse monoclonal HB65 (1:3) and polyclonal serum against FLUAV (1:1500), anti-Actin (1:2000, SCBT). Anti PKR and Phospho PKR (1:2000, Abcam), anti-FLUAV M1 HB64 (1:3) was from ATCC. Primary antibodies were incubated overnight at 4°C and incubated the next day for 1 hour with anti-HRP-conjugated secondary antibodies from GE-Healthcare (1:10000). Membranes were analyzed on a Fuji imager using the Multi Gauge 3.0 software.

Indirect immunofluorescence analysis: A549 cells were transfected with siRNA as indicated and grown on chamber slides for 72 hours. Cells were infected with MOI 5 of FPV or PR8 and fixed at the indicated time post infection with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Cells were washed twice with PBS and blocked for 30 minutes with 1% BSA. Primary and secondary antibodies were diluted in 1% BSA and incubated for 1 hour at room temperature in the dark. Cells were washed three times after each step. Anti-dsRNA J2 antibody (1:4000, engscicons, Hungary), rabbit anti-FLUAV serum (1:300) were used as primary antibodies. As secondary antibodies, Alexa 488 anti-rabbit and Alexa 594 anti-mouse (1:1000, Invitrogen) were used. Cycloheximide (75µg/ml) and actinomycin D (0.5µg/ml) were diluted from 10mg/ml stocks in normal DMEM medium and incubated with the cells from 1 hour before infection until fixation of the cells. Slides were mounted in

Fluoromount mounting medium containing DAPI (Southern biotech). Samples were analyzed with a Leica TCS SP5 microscope using the LAF software.

Expression constructs and protein purification: URH49 and UAP56 were cloned into pGEX-3x (GE Healthcare) with an N-terminal GST fusion tag (URH49for: GATAAGAATTCCGCAGAACAGGATGTGGAAAACGATC (EcoRI), rev: CTAATGAATTCAATTTACCGGCTCTGCTCGATGTATGTG (EcoRI), UAP56 for: 5' CTTATACCCGGGGCAGAGAACGATGTGGACAATG-3' (SmaI), UAP56-rev: 5'-CAATAATCCCGGGATAAACTACCGTGTCTGTTCAATGTA-3' (SmaI). Proteins were expressed in *E.coli* BL21 with 0.1mM IPTG for 4h at 32°C. Bacteria were harvested and lysed with six cycles of sonication for 30 seconds in tris lysis buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 0.1 % NP-40, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10 % glycerol, protease inhibitor cocktail (Roche)). Lysates were cleared at 12'000 g for 20 minutes and applied to a glutathione sepharose column (GE Healthcare Glutathione sepharose High Performance). Bound proteins were washed with tris wash buffer (50mM Tris-HCl pH 8.0, 100mM KCl, 0.1 % NP-40, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10 % Glycerol), eluted with tris elution buffer (20mM Tris-HCl pH 8.0, 100mM KCl, 0.1 % NP-40, 5mM $MgCl_2$, 5mM 2-mercaptoethanol, 10mM reduced glutathione, 20% glycerol) and dialysed against elution buffer without GSH. PR8-NP and FPV-NP were cloned into pQE-32 vectors (Invitrogen) containing an N-terminal 6x HIS tag, using the following primers:

PR8-NP-for: GATTATCCCGGGGCGTCTCAAGGCACCAAACGATC (SmaI),

PR8-NP-rev: CATTAGGTCGACTTATCGTATTCCTCTGCATTGTC (SalI),

FPV-NP-for: CATTACCCGGGGCGTCTCAAGGCACCAAACGATCTTATG (SmaI)

FPV-NP-rev: CGTAATCCCGGGGATTAATTAATTGTCATACTCCTCTGCATTGTCTC (SmaI). Proteins were expressed in *E.coli* M15 pRep4 with 1.5mM IPTG overnight at 22°C. Bacteria were harvested and sonicated six times for 30 seconds in tris lysis buffer containing 50mM imidazole. Lysates were cleared at 12'000 g for 20 minutes, applied to Ni-NTA columns (QIAGEN) , washed with tris

wash buffer containing 50mM imidazole), eluted with elution buffer (as described above but GSH replaced with 250mM imidazole) and dialysed against elution buffer without imidazole.

ALPHA screen: The ALPHA screen assay (Perkin Elmer) was performed in a 384-well OptiPlate with 25µl reaction volume. The recombinant proteins were incubated for 2 hours at room temperature at a final concentration of 30nM each protein in APLHA screen buffer (PBS pH 7.2, 0.1% BSA), together with AlphaLISA anti-GST acceptor beads and ALPHA screen Ni-chelate donor beads at a concentration of 20µg/ml. Interactions were analyzed in a Perkin Elmer Envision device.

Real time PCR: pCMV-UAP56 was a gift from C. Basler and P. Palese (New York), UAP56 was cloned into pcDNA3.1 (Invitrogen) with primers containing an N-terminal FLAG tag:
(For: CGAATTGGATCCGCCACCATGGACTACAAAGACGATGACGATAAA GCAGAGAACGATGTGGAC
Rev: GGATGACTCGAGGGGCGAGTCTTCTACCGTGTCTGTTCAATGTAGGAG G). FLAG-URH49 was a gift from P. Lischka (Erlangen, Germany). Nucleoproteins of PR8 and FPV were cloned into pcDNA3.1. RNA was isolated as previously described (25). Reverse transcription was performed using Superscript III (Invitrogen) according to the manufacturer`s protocol using either an oligo dT primer or a sequence specific primer (18S rRNA) and 1µg RNA. qPCR was carried out using Eva-Green ready to use qPCR mix (Biotium) and specific primers for FPV-M1, FPV-M2, FPV-NP, FPV-NS1, FPV-HA and 18S rRNA on an Applied Biosystems 7300 qPCR cycler (40x cycles 10 sec 95°C, 20 sec 55 °C, 27 sec 72°C). For the 18s rRNA control reverse transcription was performed with a 18S rRNA specific primer. Sequence detection software 1.4 was used to analyze the data. M1 for: GACCAATCCTGTCACCTC; M1 rev:

GATCCCCGTTCCCATTAAGGG; M2 for: GAGGTCGAAACGCCTAT; M2 rev: C TCCAAGCTCTATGCTGACAAA; NP for TGTGCAACATCCTCAAAGGAAA; NP rev: GAGCCACTGATCCCCTCAGA¸ NS1 for: CATGCTCATGCCCAAACAGA; NS1 rev: TCCTCGGTGAAAGCCCTTAG; HA for: GCAGGTTGATGCCAATTGC; HA rev: TTCATCCCTGTTGCCAATAATAGA;
18S rRNA for: CAAGACGGACCAGAGCGAAA; 18S rRNA rev: GGCGGGTCAT GGGAATAAC.

Northern blot: Northern blot analysis was performed as previously described (25). 10 µg of nuclear or cytoplasmic RNA were loaded per lane. After transfer of the separated RNAs onto nylon membranes the RNA was UV-crosslinked using a UV Stratalinker 1800 (Stratagene). Before hybridization the blots were stained with methylene blue in 0.3M sodium acetate pH 5.0 to visualize precursor rRNA 28S and 18S RNA. Hybridization was carried out with DIG-labeled negative-stranded RNA probes for NP and M using the Roche Northern Starter kit (Roche) according to the manufacturer's manuals. The RNA probes were *in vitro* transcribed from linearized plasmid templates containing the M and NP segment of FPV. The northern blots were analyzed on a Fuji imager. Bands representing M1 mRNA, NP mRNA or 18S rRNA were quantified using the Multi Gauge 3.0 software.

Results

The overall goal of our study was to dissect the role of UAP56 in the course of the influenza A virus replication. Since all human cells express both UAP56 and its paralogue URH49, it was pivotal to monitor effects of both helicases on influenza A virus infection. First, we tested whether both URH49 and UAP56 would interact with NP from human and/or avian influenza A virus strains. To this end, NP from the human influenza virus A/PR/8/34 (H1N1) or avian

A/FPV/Bratislawa/79/(H7N7) strain were coexpressed with FLAG-tagged human UAP56 or URH49 in human HEK 293T cells. UAP56 or URH49 were then immunoprecipitated and associated proteins analyzed by western blot. Interestingly, the NPs from both the human and avian influenza strains were capable of forming a complex with UAP56 as well as URH49 (Fig. 1A). The direct protein interaction between avian or human NP and URH49 as well as UAP56 was confirmed *in vitro* using the ALPHA screen technique. The data show that *in vitro* URH49 and UAP56 interacted with similar affinity to NP derived from a human or an avian influenza A strain (Fig 1B).

Influence of URH49 or UAP56 knockdown on viral titers

We next tested whether the individual knockdown of URH49 or UAP56 would affect the efficiency of avian and human influenza A virus replication. For this purpose A549 cells were first transfected with pools of siRNAs specific for either URH49 or UAP56. Knockdown of the targeted proteins was verified by western blot analysis (Fig. 2A). Importantly, transfection of the siRNAs did not upregulate the expression of type I interferon (IFN)-inducible protein MxA (1, 36) demonstrating that type I IFN was not induced by application of the siRNAs used in this study (Fig. 2A). Depletion of URH49 or UAP56 in A549 cells with specific siRNAs strongly affected the replication efficiency of both human and avian influenza A viruses (Fig. 2B to D). Knockdown of UAP56 or URH49 led to a reduction of avian A/FPV/Bratislawa/79(H7N7) titers by a factor of 200 and 5, respectively (Fig 2B). Similarly, we observed a significant ($p<0.05$) 5 and 50 fold titer reduction in UAP56- or URH49-depleted cells infected with a Zurich isolate of the pandemic H1N1 influenza virus (A/Zurich/8665/2009(H1N1)) (Fig 2D). Less pronounced but still significant reductions ($p<0.05$) of viral titers were observed with the laboratory strain A/PR/8/34(h1N1) were downregulation of UAP56 or URH49 led to a 20 and 5 fold decrease of viral titers (Fig. 2C). Surprisingly, infection with VSV also led to a significant ($p<0.05$) 5 and 9 fold titer

reduction at 12 hours post infection (p.i.) in UAP56 or URH49 knockdown cells, respectively (Fig 2E). To exclude the remote possibility that the observed reduction of influenza A virus titers in A549 cells depleted of URH49 or UAP56 was simply due to induction of type I interferon by siRNA treatment, we repeated the experiment in transfected A549 cells stably expressing the V-protein of simian virus 5 (SV5) (Fig. S1). The-V protein is a potent inhibitor of the type I IFN mediated expression of type I IFN-induced effector proteins (7). As expected, A549 cells stably expressing the V protein were no longer capable of synthesizing MxA upon induction with saturating amounts of IFN-α2 (Fig. S1D). Nevertheless, in these cells titers of avian influenza A virus were reduced to the same extent as in A549 wild-type cells when either URH49 or UAP56 was depleted (Fig. S1A, B, C), indicating that the observed titer reduction were not simply the result of an interferon type I mediated antiviral response.

Influence of URH49 or UAP56 knockdown on splicing and nuclear export of viral mRNAs

To better define the step(s) of the influenza A replication cycle affected by depletion of URH49 or UAP56, we examined splicing and nuclear export of viral mRNAs of segment 7 (M-segment). Digard and colleagues have recently shown that UAP56 plays a role in the nuclear export of several human influenza A mRNAs namely M1, M2, NS1 and HA mRNA while the export of other viral mRNAs, in particular NP mRNA was largely independent of UAP56 (28). A549 cells depleted of URH49 or UAP56 were infected with an MOI of 5 using avian influenza A virus for 4 hours. RNA isolated from the nuclear and cytoplasmic fraction was then analyzed either by northern blotting (Fig. 3A) using negative-stranded RNA probes specific for NP and M, or qPCR (Fig. 3B) using primers specific for M1,NP, HA and NS1 or primers specific for unspliced M1 mRNA and spliced M2 mRNA (Fig. 3E). Northern blot analysis revealed that in cells transfected with control siRNA both the mRNAs coding for NP and M1

accumulated primarily in the cytoplasm (approximately 60%) (Fig. 3A, C and D). By contrast, depletion of UAP56 or URH49 reduced nuclear export of the NP and M1 RNA to the cytoplasm. Knockdown of URH49 limited accumulation of NP mRNA and M1 mRNA to 38% and 43%, respectively (Fig. 3A, C and D). Although depletion of UAP56 had a less pronounced effect on the nuclear export of NP and M1 mRNA the data clearly indicate that both helicases are involved in this process. When the RNA samples were analyzed by qPCR very similar results were obtained (Fig. 3B). Depletion of URH49 lead to a reduced export of M1 and NP RNA in into the cytoplasm (18% M1 RNA and 19% NP RNA in the cytoplasm) when compared to treatment with control siRNA (37% M1 and 28% NP RNA in the cytoplasm), while nuclear export of HA and NS1 RNA were only marginally affected. Knockdown of UAP56 had a less pronounced effect on the nuclear export of M1 and NP (25% M1 RNA and 24% NP RNA in the cytoplasm) and again no effect on the export of HA or NS1 RNA. Furthermore, neither depletion of URH49 nor UAP56 had an influence on the ratio between the unspliced M1 and spliced M2 mRNA in the nuclear or cytoplasmic RNA fraction (Fig. 3E). The spliced M2 RNA accumulated to approximately 5% of the amount of M1 mRNA, suggesting that splicing of the M segment does not require the activity of URH49 or UAP56. However, we cannot exclude the possibility that UAP56 and URH49 have an overlapping function in assembly of the cellular splicing complex. Taken together, URH49 and to a certain extent UAP56 play a role in the nuclear export of viral mRNAs. However, the partial nuclear retention of M1 and NP in UAP56-depleted cells clearly does not explain the strong (200 fold) reduction of FPV titer in these cells.

Activation of dsRNA dependent protein kinase R (PKR)

Both UAP56 and URH49 have been previously shown to exhibit RNA helicase (unwinding) activity (35, 37). It is therefore conceivable that URH49 and/or UAP56 play a role in preventing the generation of detectable dsRNA replicative

intermediates during influenza A replication/transcription. In contrast to positive-strand RNA viruses, negative-strand RNA viruses appear not to produce detectable amounts of viral dsRNA intermediates in infected cells (39). In order to address this question, we tested the accumulation of dsRNA by monitoring the phosphorylation of the dsRNA dependent protein kinase R (PKR). Binding of dsRNAs of more than 30 base pairs in length leads to activation of the latent PKR by dimerization and autophosphorylation (29). A549 cells depleted for URH49 or UAP56 were either mock infected or infected with human and avian influenza A virus for 4 hours. Protein extracts of these cells were analyzed by western blot using a phospho PKR-specific antibody (Fig. 4A). The data (Fig. 4B and C) revealed that depletion of URH49 or UAP56 resulted in a partial activation of PKR. Moreover, influenza A virus infection of cells treated with siRNA specific for URH49 or UAP56 strongly enhanced the activation of PKR. By contrast, no activation of PKR was evident in mock infected control siRNA treated cells and only a weak activation was observed upon infection (Fig 4C). In addition, immunostaining of M1 revealed a strong reduction of M1 protein expression in UAP56 or URH49-depleted cells that paralleled the observed reduction in virus yield (Fig 2B and C). Taken together, these observations indicate that URH49 and UAP56 do indeed play a role in preventing the accumulation of dsRNA in virus infected cells. We also tested whether depletion of URH49 and UAP56 would result in the synthesis of type I interferon. This was not the case, since we did not observe activation of the transcription factor IRF-3 or secretion of interferon-β by URH49 or UAP56-depleted cells, independent of whether they were infected or not (data not shown).

Accumulation of dsRNA in UAP56-depleted cells

The observed activation of PKR in URH49 or UAP56-depleted cells infected with influenza A virus could be the result of accumulation of dsRNA. To address this question we took advantage of a monoclonal antibody highly specific for dsRNA. Weber and colleagues have previously shown that infection with

positive-stranded but not negative-stranded RNA viruses, such as influenza, resulted in the accumulation of virus-specific dsRNA in host cells (39). We infected A549 cells following treatment with either control siRNA, siRNA specific for URH49 or UAP56 with avian (Fig. 5A) or human (Fig. 5B) influenza A virus for 4 hours. As expected, in control siRNA-treated cells infection with influenza virus did not yield detectable levels of dsRNA (Fig 5A and B) In contrast, infection of UAP56-depleted cells led to a pronounced accumulation of dsRNA in the perinuclear region (Fig 5A and B). Notably, depletion of URH49 did not result in the accumulation of dsRNA in the infected cells despite the fact that we observed a considerable activiation of PKR in these cells (Fig. 4B). Mock infection of URH49-or UAP56-depleted cells yielded much lower levels of dsRNA suggesting that the observed dsRNA was predominantly of viral origin (Fig. 5C). The puzzling observation of perinuclear accumulation of dsRNA raised the possibility that the antibody detected incoming RNPs in a panhandle conformation (17) that may require unwinding before passage through the nuclear pore. To test this hypothesis, we treated the cells with actinomycin D before infection, thereby preventing *de novo* synthesis and nuclear export of viral RNAs (38). Evidently, inhibition of viral RNA synthesis strongly reduced the accumulation of dsRNA in UAP56-depleted cells. Nevertheless, small amounts of dsRNA located in the cytoplasm were detectable in UAP56-depleted and surprisingly, also in URH49-depleted cells (Fig. 6A. The fact that dsRNA accumulated very early in the infection cycle, prompted us to test whether primary transcription by the virion-associated polymerase complex (in the absence of synthesis of viral proteins) would provide sufficient amounts of viral RNA. This was clearly not the case, pretreatment of URH49- or UAP56-depleted cells with cycloheximide prevented the accumulation of dsRNA (Fig. 6B). Controls with mock infected cells only showed very low amounts of dsRNA in uninfected cells treated with UAP56 or URH49 (Figs. 5C, S2 panels A and B).

In order to assess whether the observed activity of UAP56 or URH49 may also apply to other negative-strand viruses we infected URH49- or UAP56-depleted

A549 cells with VSV and looked for the accumulation of dsRNA using the dsRNA-specific antibody. Intriguingly, similar to influenza A virus infection, dsRNA accumulated to high levels in the perinuclear region of UAP56-depleted cells whereas dsRNA was not detectable in URH49 siRNA or control siRNA treated cells (Fig. 7).

Discussion

UAP56 has been shown to link mRNA transcription and splicing to nuclear export of cellular RNA. Although encoded by two distinct genes the RNA helicases UAP56 and URH49 show an identity of 90% at the amino acid level (27). There is mounting evidence that UAP56 is involved in nuclear export of influenza virus mRNAs (2, 28, 38). This raises the question of whether or not URH49 may also play a role in transcription/replication of influenza A viruses. Co-immunoprecipitation and ALPHA screen assays have demonstrated that URH49 is capable of binding NP from both avian and human influenza A viruses and does so with similar efficiency to UAP56 (Fig. 1). However, when we analyzed the function of URH49 and UAP56 in replication of avian and human influenza virus it became evident that only UAP56 was required for efficient replication of avian influenza (Fig. 2B), while both URH49 and UAP56 showed an intermediate effect for the human influenza A virus. A possible explanation for these observations is that for human influenza the two helicases exert a partially overlapping function. In this context, it is interesting to note that depletion of URH49 or UAP56 always resulted in increased expression of the paralogous helicase (Fig. 2A). Blasts of the chicken genome database for UAP56 and URH49 revealed the presence of only the gene homologue to UAP56. Hence, it is conceivable that human URH49 may only partially complement the activity of UAP56 in conjunction with the avian influenza A replication/transcription machinery.

Several previous studies have shown that export of spliced as well as unspliced influenza mRNAs encoding late viral proteins are at least partially dependent on NFX1 and UAP56 (2, 13, 28, 38). By contrast, nuclear export of unspliced mRNAs encoding early gene products such as PB2 or NP appears to be largely independent of UAP56 viral mRNAs (28). However, in our experimental setting the export of the NP mRNA was clearly dependent on the presence of UAP56. The reason for this discrepancy between the two studies is not clear but may be explained by different cell lines and assays that were used. Further, our data demonstrate that URH49 is also involved in nuclear export of the viral NP and M1 mRNA providing further support to the notion that URH49 exerts a similar function as UAP56 although forming distinct nuclear export complexes (41).

The fact that during transcription/replication of negative-stranded RNA viruses, including influenza, dsRNA intermediates are not detectable (39) suggests the involvement of cellular RNA helicases. Hence, the association of UAP56 with vRNPs (19) may not only be crucial for nuclear export of viral mRNA but also for the prevention of formation of dsRNA replicative intermediates. The presence of dsRNA in infected cells plays a pivotal role in triggering the interferon type I response (12). This hypothesis prompted us to test whether depletion of UAP56 or URH49 (i) result in accumulation of dsRNA and (ii) would lead to the activation of the dsRNA-dependent PKR. Since PKR is localized in the cytoplasm its pronounced activation in UAP56- or URH49-depleted cells pointed to the accumulation of dsRNA in the cytoplasm. Immunostaining of dsRNA using a specific antibody revealed that at least for UAP56 this was indeed the case (Fig 5). Infection of UAP56-depleted cells with avian and human influenza A virus or VSV invariably led to the accumulation of dsRNA in the perinuclear region (Fig. 4 and 6) thus providing an explanation for the activation of PKR. However, in the case of URH49 depletion, it is not clear why no dsRNA was detectable, despite the pronounced activation of PKR in these cells. A possible explanation for this finding may be that only small amounts of dsRNA were produced that were sufficient for the activation of PKR but not detectable by the anti-dsRNA antibody.

Alternatively, it is conceivable that UAP56 may be able to complement the unwinding activity of URH49 in the cytoplasm but not vice versa. So far a defined activity in the cytoplasm has only been demonstrated for UAP56 (20, 31).

Recently, Burgui and colleages have shown that the influenza polymerase complex remains associated with the cap structure of the viral mRNA in the cytoplasm substituting for the function of the cellular factor eIF4E (3). Hence, it is tempting to speculate that UAP56 remains associated with the viral mRNA via the polymerase complex facilitating translation of the viral mRNA. Indeed, a function similar to the activity of eIF4A in protein translation has been recently shown for UAP56 in cardiomyocytes (30).

Although, it is highly likely that the observed dsRNA is predominantly of viral origin we have no formal proof. Nevertheless, inhibition of the replication with actinomycin D or cycloheximide (allowing primary transcription) abrogated the formation of dsRNA. Recently, Dauber and colleagues have shown that in the absence of a functional NS1 protein cytoplasmic vRNPs are capable of inducing dsRNA-dependent PKR during the late phase of influenza B infection (5). Incidentally, B/NS1 has been reported to colocalize with several factors of the nuclear export machinery in nuclear speckles and to interact with UAP56 *in vitro* (32). However, inhibition of the CRM-1 mediated nuclear export of vRNPs by treatment with leptomycin had no effect on the accumulation of dsRNA (data not shown) strongly suggesting that the accumulating dsRNA in the perinuclear region does not consist of vRNPs (8).

There is ample evidence that the NS1 protein of influenza viruses efficiently interfere with the activation of PKR (reviewed in (11, 40). Hence, the observed activation of PKR in influenza virus infected cells lacking either UAP56 or URH49 could be due to low levels of NS1. Nuclear export of NS1 mRNA is reduced in cells depleted of UAP56 (28) or URH49 (data not shown). However, despite a pronounced activation of PKR, depletion of UAP56 or URH49 did not result in the synthesis of type I interferons in influenza virus infected cells. Neither did we observe activation of IRF3, nor were we able to detect even minute amounts of

interferon-β in culture supernatants (data not shown). It is conceivable that the observed inhibition of the interferon signalling cascade is due to the activity of low levels of NS1 interfering with activation of RIG-1 (10, 40) suggesting that the reduced amount of NS1 is still sufficient for interfering with the interferon type I response. In this context, it is interesting to note that Ostertag and colleagues reported that infection of host cells with a polR mutant of VSV (containing a single amino acid substitution in the N protein) resulted in the overproduction of viral dsRNA, and as a consequence, activation of PKR. Surprisingly however, the observed growth restriction of this mutant virus was independent of the interferon type I response (23, 24). Hence, it would be interesting to test whether the observed growth restriction phenotype of this VSV polR mutant could be linked to the activity of UAP56.

It is intriguing that influenza-induced dsRNA was not observed in the nuclei of UAP56- nor URH49-depleted cells (Fig. 5). Since influenza viruses replicate in the host cell nucleus we would have expected accumulation of dsRNA primarily in the nucleus. One possible explanation is that URH49 and UAP56 may be able to complement their unwinding activity in the nucleus. Unfortunately, it is not possible to test this hypothesis since concomitant depletion of URH49 and UAP56 led to cell death within 72 hours of the start of siRNA treatment (data not shown) (see also (14, 15)).

Taken together, we were able to show that the cellular RNA helicases UAP56 and URH49 not only play a role in nuclear export of viral RNA but also prevent the formation of dsRNA and hence, the antiviral response of PKR in influenza virus and VSV-infected cells. Moreover, it will be interesting to elucidate whether the usurpation of activity of UAP56 may be a general viral strategy for evading the innate immune system.

Acknowledgements

We thank Alexandra Trkola, Anders Krarup and Axel Mann for helpful discussions. The cDNA for UAP56 was kindly provided by C. Basler and P. Palese. The FLAG-URH49 expression construct was kindly provided by T. Stamminger. This work was supported by a grant from the Swiss National Science foundation.

Figures manuscript 1

Figure 1

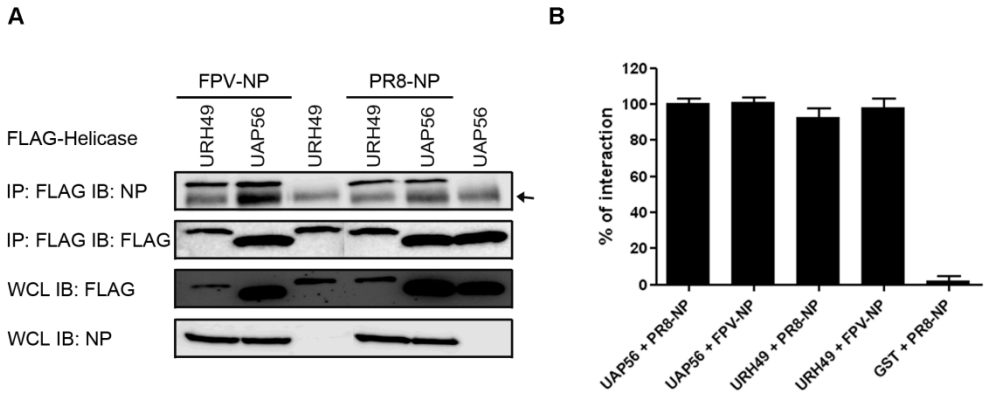

FIG. 1. Human and avian influenza A virus nucleoprotein interact with cellular helicases UAP56 and URH49. (A) Co-immunoprecipitation assays were performed with FLAG-tagged UAP56 or URH49 and avian or human influenza A virus nucleoproteins. The arrow indicates the position of the heavy chain of the antibodies used for immunoprecipitation. (B) Affinity purified GST-UAP56 or GST-URH49 and HIS-PR8-NP (A/PR8/34) or HIS-FPV-NP (A/FPV/Bratislava/79) were mixed and assayed for (30nM of each protein) interaction *in vitro* using the ALPHA screen technology. As a negative control GST alone was incubated with HIS-PR8-NP. The interaction of GST-UAP56 and HIS-PR8-NP was set to 100% as a reference for the other interactions. (IP: immunoprecipitation, IB: immunoblot, WCL: whole cell lysate).

Figure 2

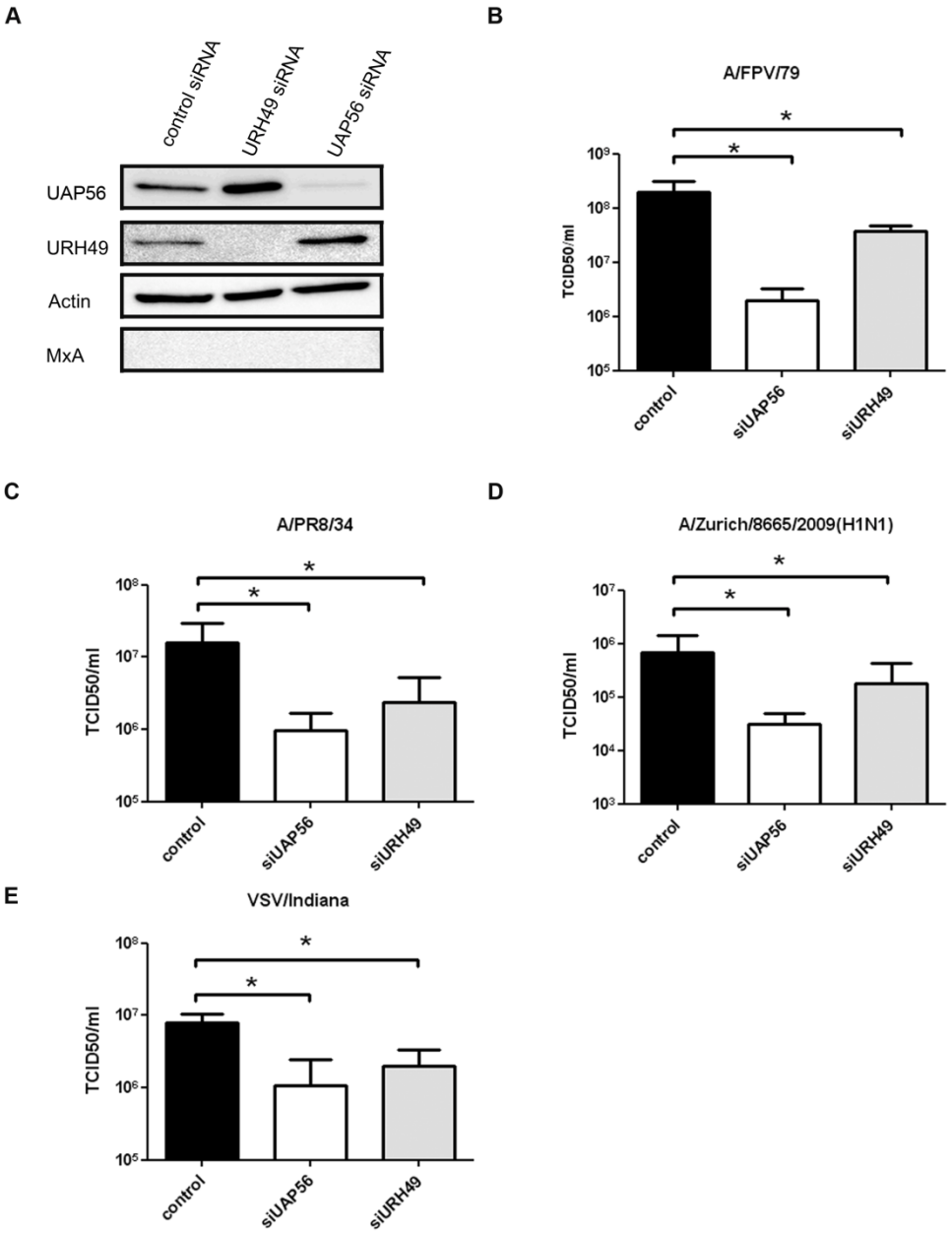

FIG. 2. Knockdown of UAP56 or URH49 results in a strong titer reduction of influenza A virus. (A) A549 cells were treated with the indicated siRNAs for 48 hours. Protein levels of UAP56 and URH49 were analyzed by western blot. MxA was used to monitor induction of the interferon in siRNA treated cells. A549 cells were treated with the indicated siRNAs for 48 hours and subsequently infected with (B) FPV (MOI = 0.1) (C) PR8 (MOI = 0.1) (D) A/Zurich/8665/2009(H1N1) (MOI=1) (E) VSV Indiana (MOI = 0.1), culture supernatants were taken 24 hours p.i. and assayed for virus titer using the TCID50 method, for VSV virus titers were measured after 12h. * indicates $p < 0.05$

Figure 3

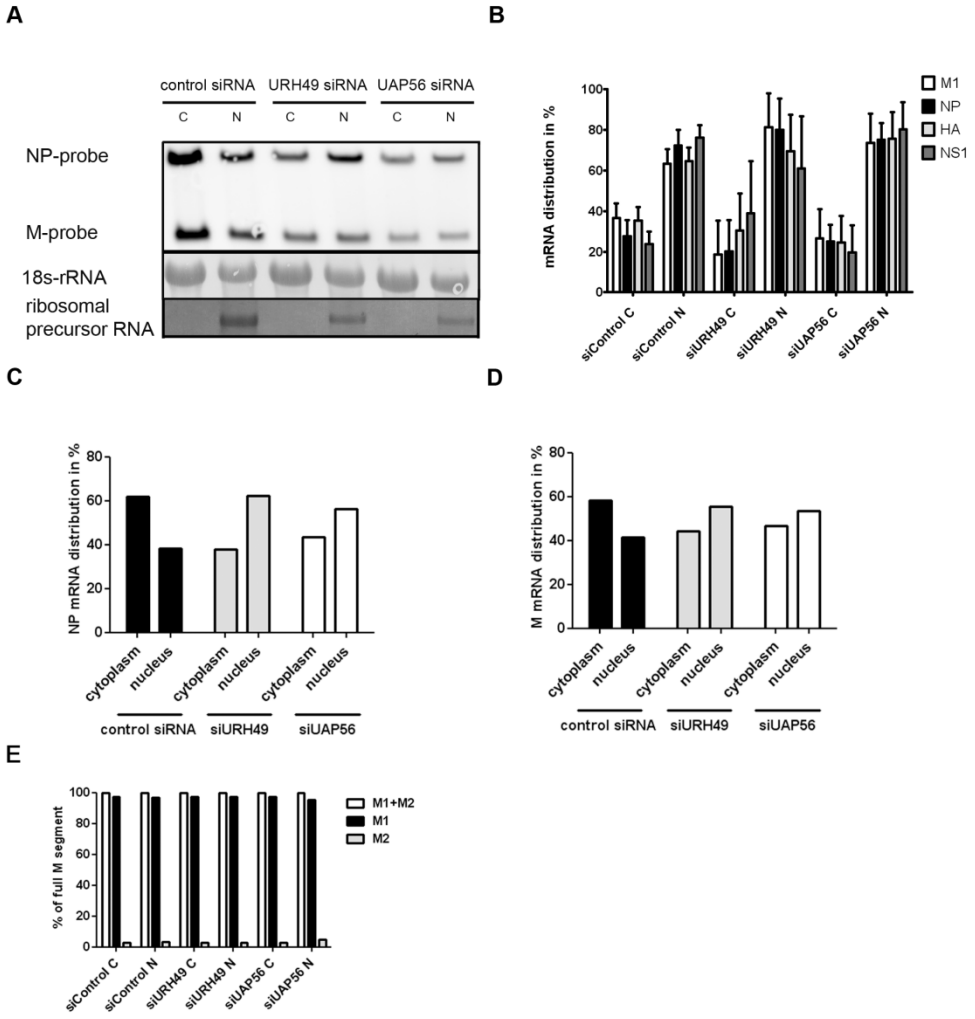

Figure 3. Reduced export of viral mRNAs in cells treated with UAP56 or URH49-specific siRNA upon infection with influenza A virus. (A) A549 cells were treated with the indicated siRNA for 72 hours and subsequently infected with FPV for 4 hours (MOI = 5). Cytoplasmic and nuclear RNAs were isolated, 5 µg per lane of RNA were analyzed by northern blot. Methylene blue-stained 18S rRNA was used to normalize total RNA amounts in each lane. Precusor rRNA was only detectable in the nuclear RNA fraction. The separated RNAs were hybridized with DIG-labeled probes specific for influenza A virus M and NP segment. (B) Reverse transcription was performed from cytoplasmic and nuclear RNA using oligodT primer and 1µg RNA, M1, NP, HA and NS1 levels in the two subcellular fractions were analyzed by qPCR using specific primer pairs for the indicated viral mRNAs, results are shown as a mean of five independent experiments. Nuclear or cytoplasmic localization of NP mRNA (C) and M1 mRNA (D) were quantified measuring band intensities using the Multi Gauge software from Fujifilm. Total mRNA from the cytoplasmic and nuclear fraction of control siRNA-treated cells was set as the reference. (E) Splicing of the influenza A virus M segment was not affected by knockdown of UAP56 or UARH49. Reverse transcription was performed from cytoplasmic and nuclear RNA using oligodT primer and 1 µg RNA, M1 and M2 levels were assessed by real-time qPCR using specific primer pairs for M1 and M2.

Figure 4

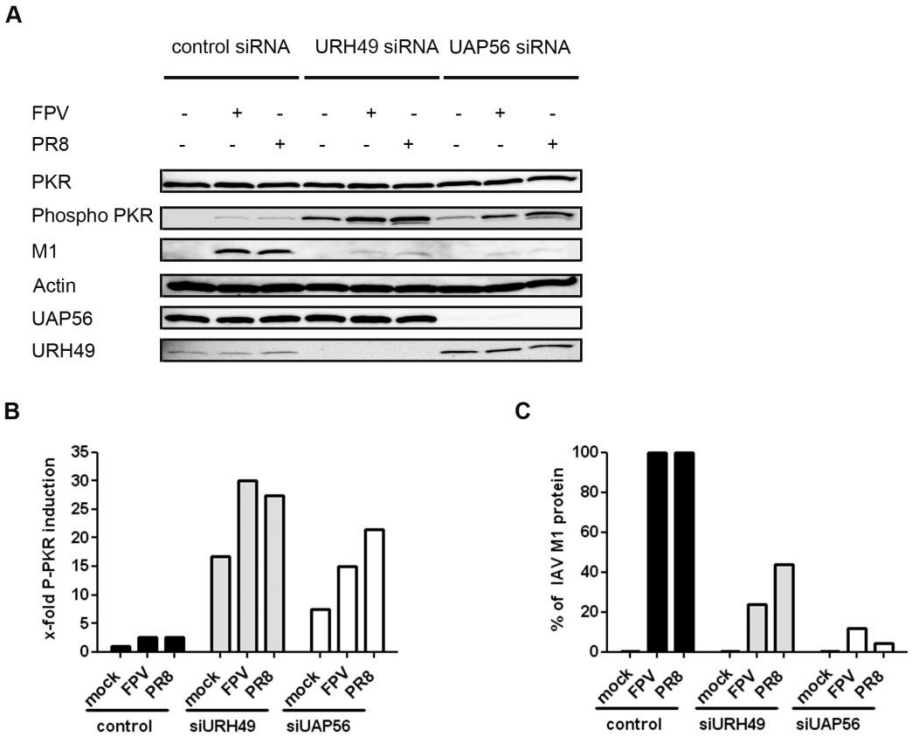

FIG. 4. PKR is phosphorylated in UAP56 and URH49 knockdown cells upon infection with influenza A virus. (A) A549 cells were treated with the 30nM of the indicated siRNAs for 72 hours and subsequently infected with influenza PR8 or FPV for 4 hours (MOI = 5). Total cell lysates were analyzed by western blotting using specific antibodies against PKR, phospho PKR, influenza M1, actin, UAP56 and URH49. (B) Phospho PKR levels (C) and influenza M1 protein levels were quantified measuring band intensities using the Multi Gauge software from Fujifilm.

Figure 5

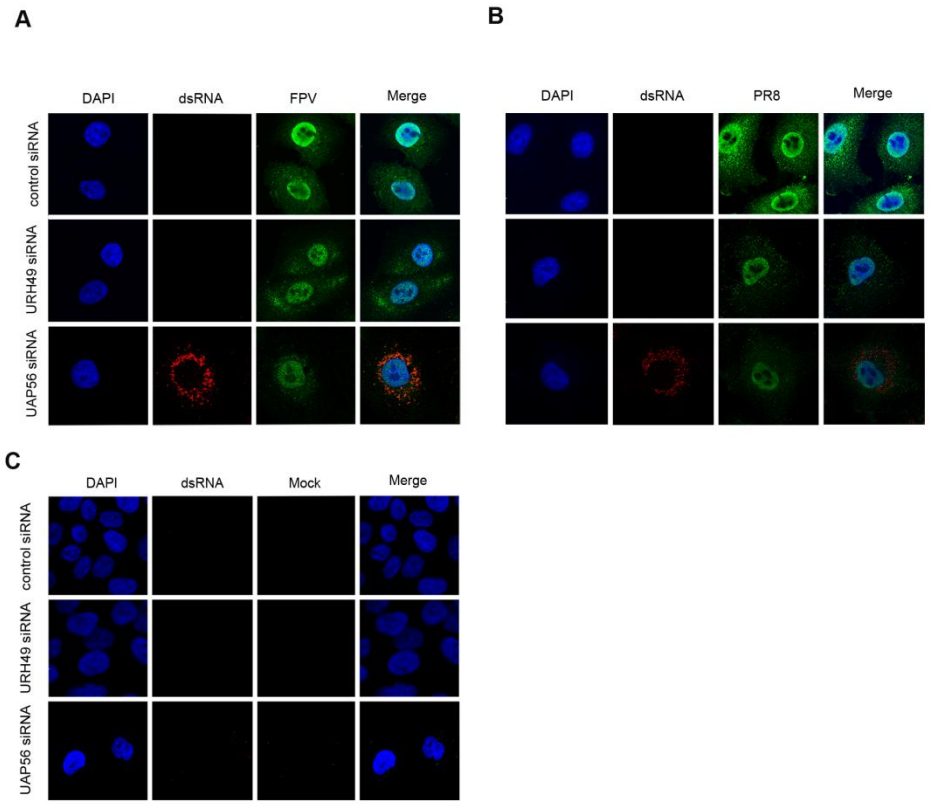

FIG. 5. dsRNA accumulates in the perinuclear region of UAP56 knockdown cells infected with human or avian influenza A virus. A549 cells were treated with the indicated siRNAs for 72 hours and infected with FPV (A) ,PR8 (B) for 4 hours and mock infected (C). Cells were fixed with 4% formaldehyde and permeabilzed with 0.5% Triton X-100. Cells were stained using an anti-influenza serum (1:300) and anti-dsRNA antibody (1:3000). Pictures were taken with a Leica TCS-SP5 confocal microscope.

Figure 6

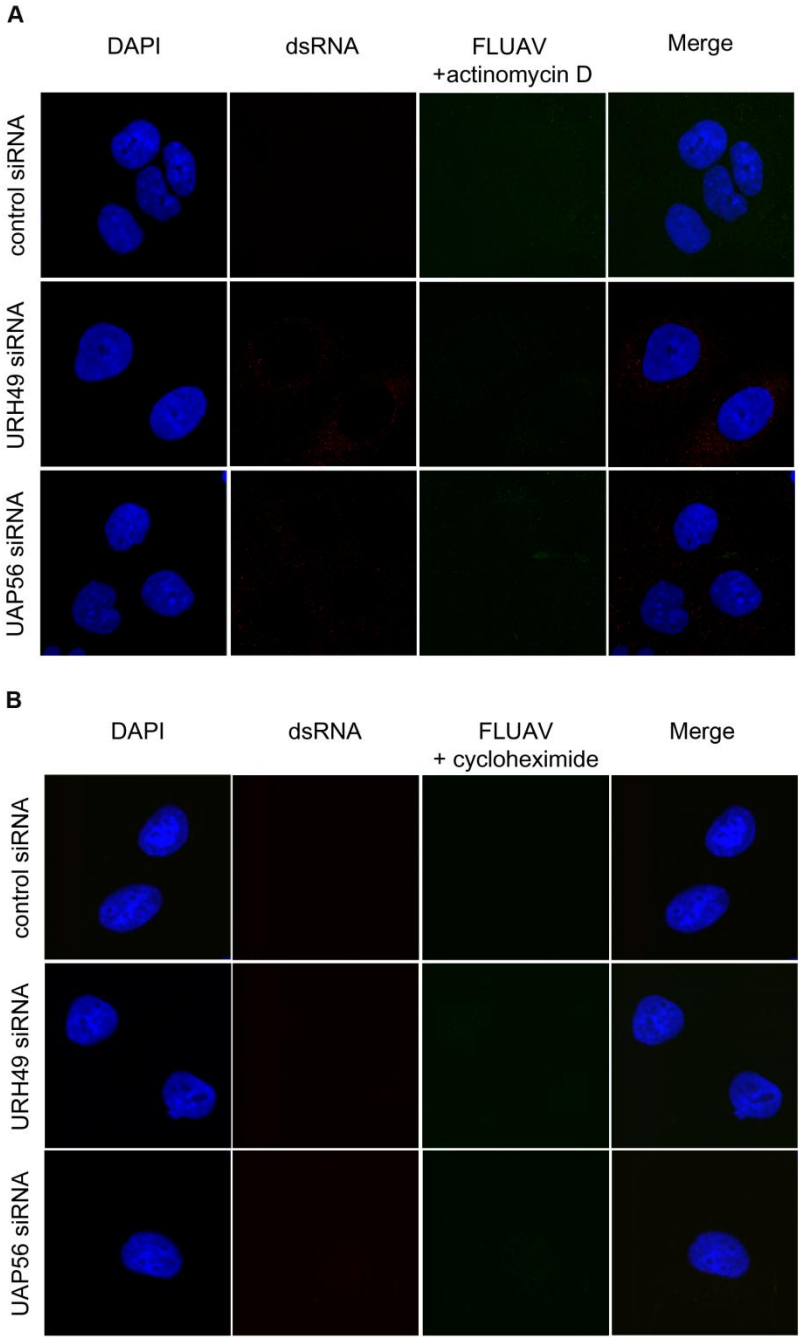

FIG. 6. Treatment with cycloheximide or actinomycin D prevents accumulation of dsRNA in UAP56-depleted cells infected with influenza A virus. A549 cells were treated with the indicated siRNAs for 72 hours. Cells were incubated with (A) actinomycin D (0.5 µg/ml) or (B) cycloheximide (75 µg/ml) 1 hour before infection. Cells were infected with FPV (MOI = 5). Inhibitors remained in the media during and after infection. Cells were fixed 4 hours post infection and stained using an anti-influenza serum (1:300) and anti-dsRNA antibody (1:3000). Pictures were taken with a Leica TCS-SP5 confocal microscope.

Figure 7

A

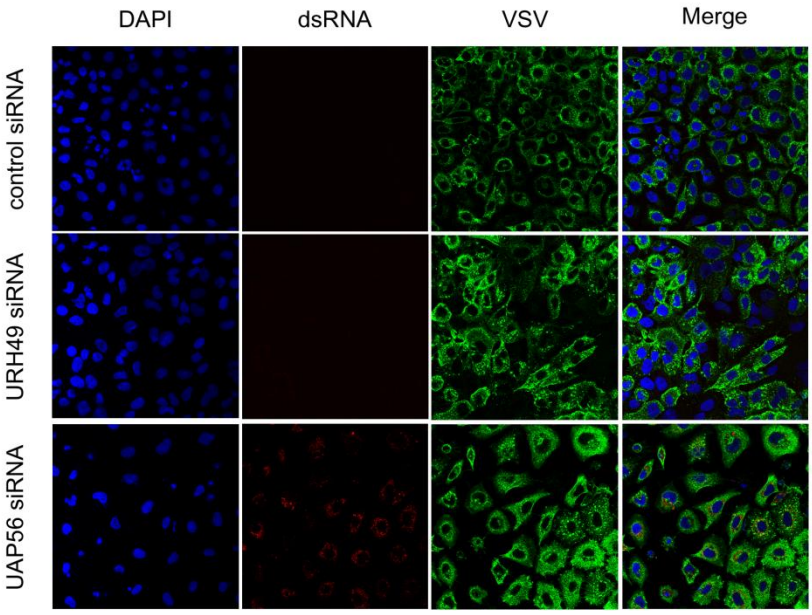

FIG. 7. Vesicluar stomatitis virus induces accumulation of dsRNA in UAP56 knock-down cells. (A) A549 cells were treated with the indicated siRNAs for 72 hours and subsequently infected with VSV Indiana for 4 hours. Cells were fixed with 4% formaldehyde and permeabilzed with 0.5% Triton X-100. Cells were stained using a polyclonal anti-VSV serum (1:300) and anti-dsRNA antibody (1:3000). Pictures were taken with a Leica TCS-SP5 confocal microscope.

Supplementary 1

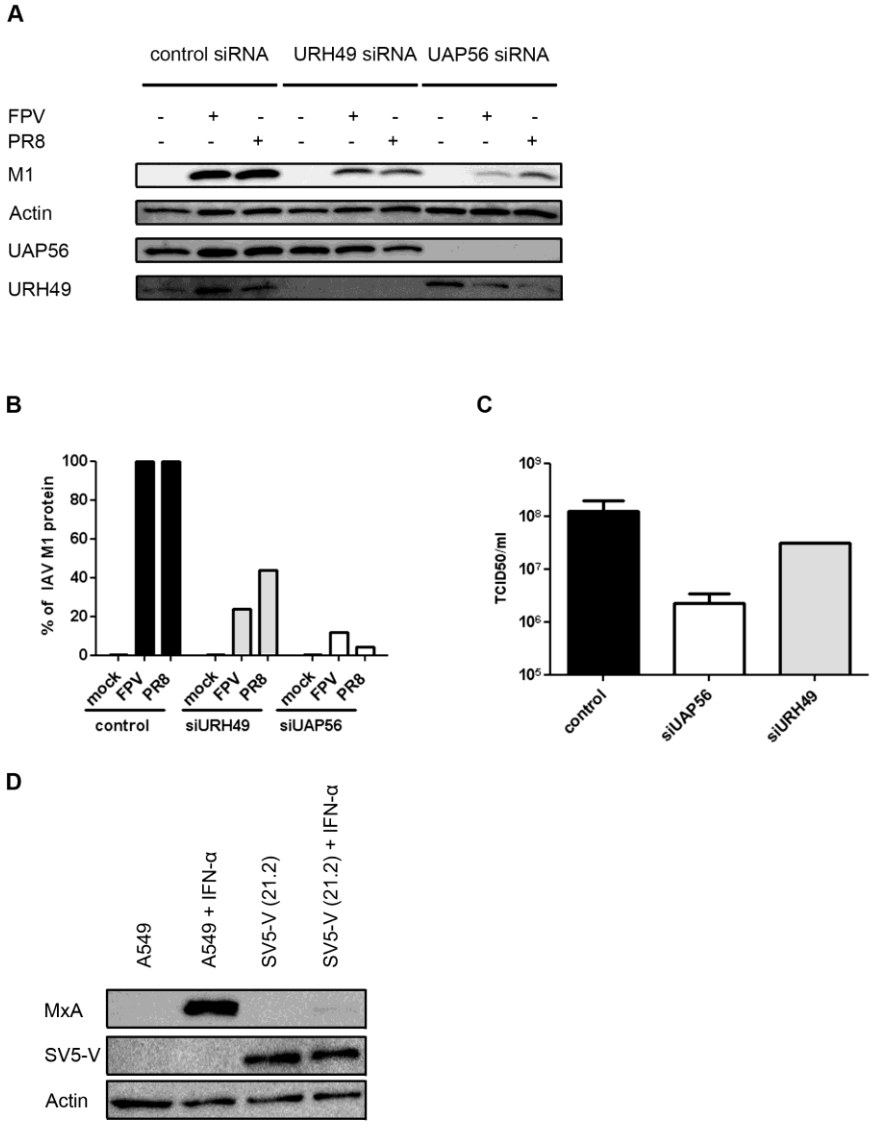

FIG. S1. A549 cells stable expressing the Simian Virus 5 V protein were treated with the indicated siRNAs for 72 hours and infected with Influenza PR8 and FPV for 4h (MOI = 5). Total cell lysates were analyzed by western blots using specific antibodies against Influenza M1, actin, UAP56 and URH49. (A) Influenza M1 protein levels (B) were quantified using the Multi Gauge software from Fujifilm. (C) A549-SV5-V cells were treated with siRNAs for 48 hours and subsequently infected with FPV. 24 hours p.i. virus yields in the culture supernatants were determined by the TCID50 method. As a control A549-SV5-V cells were stimulated with interferon-α and MxA was used as a marker for interferon-induced proteins. (D) Cells expressing the SV5-V protein show a very weak expression of MxA, indicating that most cells express functional SV5-V protein.

Supplementary 2

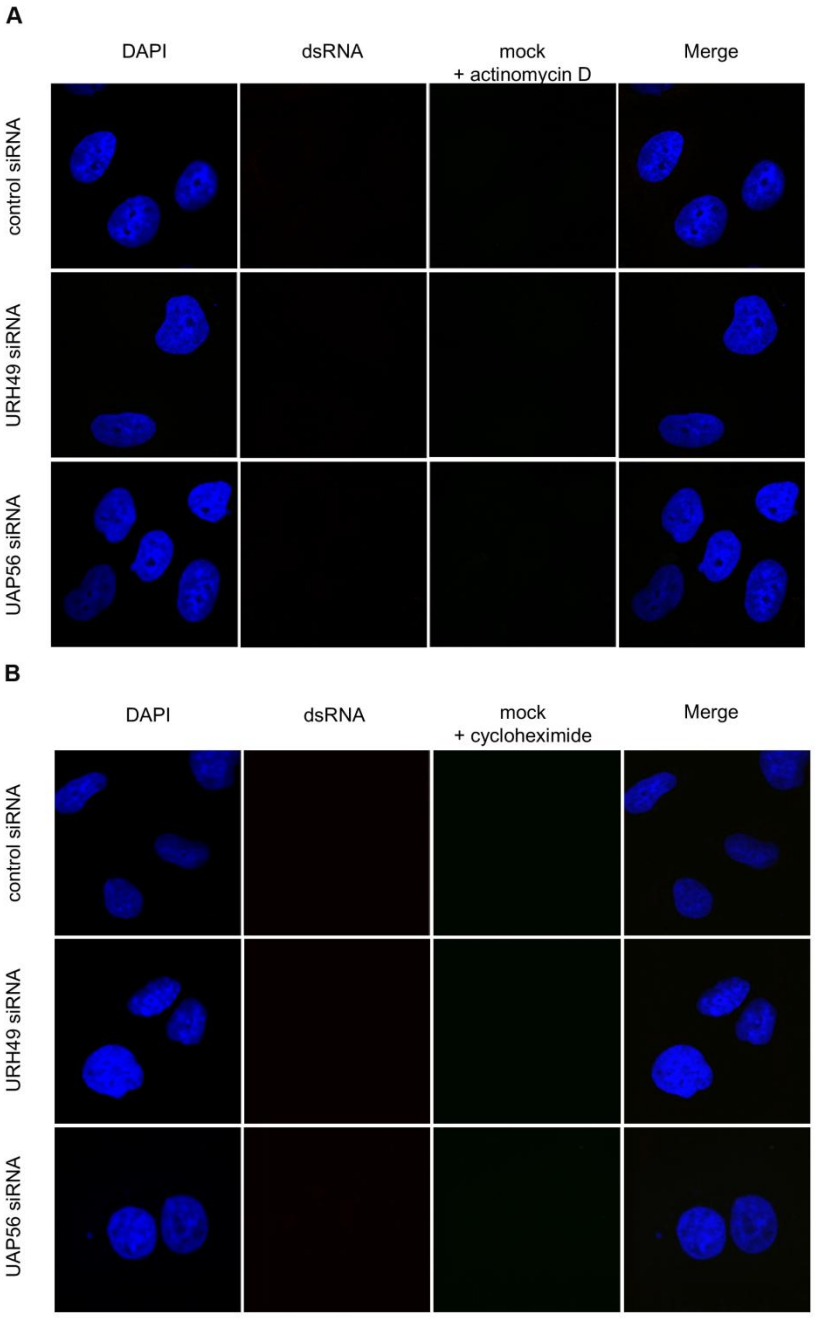

FIG. S2. Treatment with cycloheximide or actinomycin D has no effect in mock infected cells. A549 cells were treated with the indicated siRNAs for 72 hours. Cells were incubated with (A) actinomycin D (0.5µg/ml) and (B) cycloheximide (75µg/ml) 1 hour before mock infection. Inhibitors remained in the media during and after mock infection. After 4 hours cells were fixed and immunostained using an anti-influenza A serum (1:300) and anti-dsRNA antibody J2 (1:3000). Pictures were taken with a Leica TCS-SP5 confocal microscope.

References

1. Aebi, M., J. Fah, N. Hurt, C. E. Samuel, D. Thomis, L. Bazzigher, J. Pavlovic, O. Haller, and P. Staeheli. 1989. cDNA structures and regulation of two interferon-induced human Mx proteins. Mol Cell Biol 9:5062-72.
2. Amorim, M. J., E. K. Read, R. M. Dalton, L. Medcalf, and P. Digard. 2007. Nuclear export of influenza A virus mRNAs requires ongoing RNA polymerase II activity. Traffic 8:1-11.
3. Burgui, I., E. Yanguez, N. Sonenberg, and A. Nieto. 2007. Influenza virus mRNA translation revisited: is the eIF4E cap-binding factor required for viral mRNA translation? J Virol 81:12427-38.
4. Carmody, S. R., and S. R. Wente. 2009. mRNA nuclear export at a glance. J Cell Sci 122:1933-7.
5. Dauber, B., L. Martinez-Sobrido, J. Schneider, R. Hai, Z. Waibler, U. Kalinke, A. Garcia-Sastre, and T. Wolff. 2009. Influenza B virus ribonucleoprotein is a potent activator of the antiviral kinase PKR. PLoS Pathog 5:e1000473.
6. Dias, A., D. Bouvier, T. Crepin, A. A. McCarthy, D. J. Hart, F. Baudin, S. Cusack, and R. W. Ruigrok. 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458:914-8.
7. Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall. 1999. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. J Virol 73:9928-33.
8. Elton, D., M. Simpson-Holley, K. Archer, L. Medcalf, R. Hallam, J. McCauley, and P. Digard. 2001. Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway. J Virol 75:408-19.
9. Fleckner, J., M. Zhang, J. Valcarcel, and M. R. Green. 1997. U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction. Genes Dev 11:1864-72.
10. Gack, M. U., R. A. Albrecht, T. Urano, K. S. Inn, I. C. Huang, E. Carnero, M. Farzan, S. Inoue, J. U. Jung, and A. Garcia-Sastre. 2009. Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe 5:439-49.
11. Hale, B. G., R. E. Randall, J. Ortin, and D. Jackson. 2008. The multifunctional NS1 protein of influenza A viruses. J Gen Virol 89:2359-76.
12. Haller, O., G. Kochs, and F. Weber. 2006. The interferon response circuit: induction and suppression by pathogenic viruses. Virology 344:119-30.
13. Hao, L., A. Sakurai, T. Watanabe, E. Sorensen, C. A. Nidom, M. A. Newton, P. Ahlquist, and Y. Kawaoka. 2008. Drosophila RNAi screen identifies host genes important for influenza virus replication. Nature 454:890-3.
14. Hautbergue, G. M., M. L. Hung, M. J. Walsh, A. P. Snijders, C. T. Chang, R. Jones, C. P. Ponting, M. J. Dickman, and S. A. Wilson. 2009. UIF, a New mRNA export adaptor that works together with REF/ALY, requires FACT for recruitment to mRNA. Curr Biol 19:1918-24.

15. Kapadia, F., A. Pryor, T. H. Chang, and L. F. Johnson. 2006. Nuclear localization of poly(A)+ mRNA following siRNA reduction of expression of the mammalian RNA helicases UAP56 and URH49. Gene 384:37-44.
16. Lamb, R. A., and C. M. Horvath. 1991. Diversity of coding strategies in influenza viruses. Trends Genet 7:261-6.
17. Luo, G. X., W. Luytjes, M. Enami, and P. Palese. 1991. The polyadenylation signal of influenza virus RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. J Virol 65:2861-7.
18. Luo, M. L., Z. Zhou, K. Magni, C. Christoforides, J. Rappsilber, M. Mann, and R. Reed. 2001. Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. Nature 413:644-7.
19. Mayer, D., K. Molawi, L. Martinez-Sobrido, A. Ghanem, S. Thomas, S. Baginsky, J. Grossmann, A. Garcia-Sastre, and M. Schwemmle. 2007. Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches. J Proteome Res 6:672-82.
20. Meignin, C., and I. Davis. 2008. UAP56 RNA helicase is required for axis specification and cytoplasmic mRNA localization in Drosophila. Dev Biol 315:89-98.
21. Momose, F., C. F. Basler, R. E. O'Neill, A. Iwamatsu, P. Palese, and K. Nagata. 2001. Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis. J Virol 75:1899-908.
22. Munoz, F. M., G. J. Galasso, J. M. Gwaltney, Jr., F. G. Hayden, B. Murphy, R. Webster, P. Wright, and R. B. Couch. 2000. Current research on influenza and other respiratory viruses: II international symposium. Antiviral Res 46:91-124.
23. Ostertag, D., T. M. Hoblitzell-Ostertag, and J. Perrault. 2007. Cell-type-specific growth restriction of vesicular stomatitis virus polR mutants is linked to defective viral polymerase function. J Virol 81:492-502.
24. Ostertag, D., T. M. Hoblitzell-Ostertag, and J. Perrault. 2007. Overproduction of double-stranded RNA in vesicular stomatitis virus-infected cells activates a constitutive cell-type-specific antiviral response. J Virol 81:503-13.
25. Pavlovic, J., O. Haller, and P. Staeheli. 1992. Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. J Virol 66:2564-9.
26. Pavlovic, J., T. Zurcher, O. Haller, and P. Staeheli. 1990. Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. J Virol 64:3370-5.
27. Pryor, A., L. Tung, Z. Yang, F. Kapadia, T. H. Chang, and L. F. Johnson. 2004. Growth-regulated expression and G0-specific turnover of the mRNA that encodes URH49, a mammalian DExH/D box protein that is highly related to the mRNA export protein UAP56. Nucleic Acids Res 32:1857-65.
28. Read, E. K., and P. Digard. Individual influenza A virus mRNAs show differential dependence on cellular NXF1/TAP for their nuclear export. J Gen Virol 91:1290-301.
29. Sadler, A. J., and B. R. Williams. 2007. Structure and function of the protein kinase R. Curr Top Microbiol Immunol 316:253-92.
30. Sahni, A., N. Wang, and J. D. Alexis. 2010. UAP56 is an important regulator of protein synthesis and growth in cardiomyocytes. Biochem Biophys Res Commun 393:106-10.
31. Schmidt, U., K. B. Im, C. Benzing, S. Janjetovic, K. Rippe, P. Lichter, and M. Wachsmuth. 2009. Assembly and mobility of exon-exon junction complexes in living cells. RNA 15:862-76.

32. Schneider, J., B. Dauber, K. Melen, I. Julkunen, and T. Wolff. 2009. Analysis of influenza B Virus NS1 protein trafficking reveals a novel interaction with nuclear speckle domains. J Virol 83:701-11.
33. Shen, H. 2009. UAP56- a key player with surprisingly diverse roles in pre-mRNA splicing and nuclear export. BMB Rep 42:185-8.
34. Shen, H., X. Zheng, J. Shen, L. Zhang, R. Zhao, and M. R. Green. 2008. Distinct activities of the DExD/H-box splicing factor hUAP56 facilitate stepwise assembly of the spliceosome. Genes Dev 22:1796-803.
35. Shen, J., L. Zhang, and R. Zhao. 2007. Biochemical characterization of the ATPase and helicase activity of UAP56, an essential pre-mRNA splicing and mRNA export factor. J Biol Chem 282:22544-50.
36. Simon, A., J. Fah, O. Haller, and P. Staeheli. 1991. Interferon-regulated Mx genes are not responsive to interleukin-1, tumor necrosis factor, and other cytokines. J Virol 65:968-71.
37. Sugiura, T., Y. Nagano, and Y. Noguchi. 2007. DDX39, upregulated in lung squamous cell cancer, displays RNA helicase activities and promotes cancer cell growth. Cancer Biol Ther 6:957-64.
38. Wang, W., Z. Q. Cui, H. Han, Z. P. Zhang, H. P. Wei, Y. F. Zhou, Z. Chen, and X. E. Zhang. 2008. Imaging and characterizing influenza A virus mRNA transport in living cells. Nucleic Acids Res 36:4913-28.
39. Weber, F., V. Wagner, S. B. Rasmussen, R. Hartmann, and S. R. Paludan. 2006. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol 80:5059-64.
40. Wolff, T., and S. Ludwig. 2009. Influenza viruses control the vertebrate type I interferon system: factors, mechanisms, and consequences. J Interferon Cytokine Res 29:549-57.
41. Yamazaki, T., N. Fujiwara, H. Yukinaga, M. Ebisuya, T. Shiki, T. Kurihara, N. Kioka, T. Kambe, M. Nagao, E. Nishida, and S. Masuda. 2010. The closely related RNA helicases, UAP56 and URH49, preferentially form distinct mRNA export machineries and coordinately regulate mitotic progression. Mol Biol Cell 21:2953-65.

Manuscript 2

The interferon-induced antiviral protein MxA interacts with the cellular RNA helicases UAP56 and URH49

The interferon-induced antiviral protein MxA interacts with the cellular RNA helicases UAP56 and URH49

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Contributions: C.W. and J.P designed the experiments. C.W performed all experiments, ALPHA screens were performed by C.W and D.M. Confocal microscopy was done by C.W. with the help of T.L. Split GFP experiments were carried out by C.W., J.P. and E.M.

Abstract

Mx proteins are a family of large GTPases, which are exclusively induced by interferon-α/β and have a broad antiviral activity against several viruses, including influenza A Virus (IAV), vesicular stomatitis (VSV) virus, thogotvirus (THOV) and La Crosse virus (LACV). Although the antiviral activity of human MxA has been studied extensively, only a very limited number of cellular factors have been described which directly interact with human MxA and the exact mechanism remains unclear and has to be further elucidated. We examined whether MxA directly interacts with two cellular helicases, UAP56 and URH49, which are involved in spliceosome assembly and nuclear mRNA export. UAP56 and URH49 were recently shown to be important for IAV replication preventing the formation of dsRNA as well as leading to a nuclear accumulation of IAV segment specific mRNAs in knockdown cells for UAP56. Our data shows a direct interaction between MxA and UAP56 or URH49, which we could show endogenously as well as *in vitro* using ALPHA screen assays. Upon interaction we observed a translocation of UAP56 and URH49 into the cytoplasm of MxA expressing cells. Moreover, we established a split GFP system for MxA and UAP56 to confirm our interaction as well as the intracellular relocalization upon the interaction of MxA and the helicases. Taken together, we show a direct interaction for MxA and the two cellular helicases UAP56 and URH49, giving new insights into the molecular mechanism of human MxA.

Introduction

Mx proteins are a family of large GTPases, including dynamin, which can be found in most vertebrates. They play a pivotal role in the type I interferon mediated response against a broad range of viral infections [1]. Mx proteins share a high intrinsic GTPase activity and the ability to form large oligomeric structures [2-5]. The cytoplasmic human MxA has been described to inhibit several RNA and DNA viruses including influenza A virus (IAV), measles virus, vesicular stomatitis virus (VSV) or semliki forest virus [6-9]. Recently the crystal structure of the stalk region of MxA has been solved and in combination with similar structural data from dynamin a model for a four helical bundle formation of MxA was proposed [10]. MxA mutants lacking the ability to form oligomers were shown to have no antiviral activity against IAV [10]. Although the antiviral function of MxA has been studied extensively, little is known about the exact molecular mechanism of inhibition. MxA inhibits thogotovirus (THOV) infection via a physical interaction with the THOV nucleocapsid, blocking the nuclear import of THOV nucleocapsids [11]. For LACV an interaction between MxA and the LACV nucleoprotein was shown, which is sequestered by MxA into perinuclear regions [12]. MxA exhibits a typical granular staining pattern in the cytoplasm and associates partially with the smooth endoplasmic reticulum, suggesting a role in cellular trafficking [13]. However, for IAV no direct interaction of Mx-proteins with any viral protein could be shown, suggesting that cellular factors might be involved exerting a bridging function between MxA and IAV proteins. Momose and colleagues reported an interaction between the cellular DEAD box helicase UAP56 and IAV nucleoprotein (NP), which leads to an increased vRNA synthesis *in vitro* [14]. UAP56 plays an important role in the assembly of the spliceosome and in nuclear export of spliced and unspliced mRNA out of the nucleus. It has been first described as an essential splicing factor required for spliceosome assembly [15-17]. UAP56 is part of the transcription-export complex (TREX) and recruits the adaptor protein Aly/REF to the exon-junction complex (EJC) bound to

pre-mRNAs and subsequently guides the pre-mRNAs to the nuclear transcription factor (NFX1) resulting in nuclear export of the mRNAs [16, 19, 20]. Recently, a close paralogue of UAP56 termed UAP56-related helicase, 49kDa (URH49) has been identified that has 90% amino acid identity and exhibits similar cellular functions. Like UAP56, URH49 is able to bind Aly/REF and is able to complement the homologue Sub2p, in yeast [21]. Until now, no data is available for URH49 to be involved in spliceosome assembly, although it was shown for UAP56 and URH49 siRNA knockdowns to show a poly(A)+ RNA retention phenotype in the nucleus [22]. Knockdown of both helicases at the same time leads to cell death within 72 hours [22]. UAP56 and URH49 have been shown to be primarily located in the nucleus, accumulating in RNA-splicing speckled domains of the nucleus and nearby nuclear regions, although UAP56 was also reported to partially shuttle to the cytoplasm [20, 23, 24]. Furthermore, we recently demonstrated that UAP56 and URH49 are required for efficient IAV replication and prevention of dsRNA formation in cells infected with IAV (Wisskirchen et al).

In this study, we addressed the question whether human MxA interacts with cellular factors, in particular with the cellular helicases UAP56 and URH49. We observed an interaction between human MxA or murine Mx1 and/or UAP56/URH49, using co-immunoprecipitations and ALPHA screen assays. In addition, we observed a change in the distribution pattern of UAP56 and URH49 in cells expressing MxA, indicating a colocalization of the proteins under MxA expressing conditions, as well as showing a dominant-negative effect of UAP56 or URH49 on an IAV mini-replicon system, which is comparable to the effect of MxA on IAV replication. Finally, we established a split GFP system to assess the direct interaction UAP56/URH49 and MxA using different constructs in living cells and trying to determine the exact subcellular localization of the interactions.

Materials and methods

Cells and transfections: A549, 3T3 and HEK 293T (ATCC) cells were cultured in DMEM (Gibco) supplemented with 10% FCS, 1% Pen/Strep (Gibco) and 1% Glutamax (Gibco). Cells were transfected at 80% confluency with jetPEI transfection reagent (Polyplus) according to the manual and incubated for 24 hours before being analyzed.

Influenza A replicon system: The cDNAs of the viral polymerase subunits (PA, PB1 and PB2) and the viral nucleoprotein (NP) are derived from influenza A/Thailand/(KAN-1)/2004(H5N1) virus. 293T cells were seeded in 12-well plates and transfected with jetPEI (Polyplus). 10ng of plasmid were transfected for PB1, PB2 and PA and 100ng for NP together with 50ng of plasmid pPol-Luc-RT carrying the firefly luciferase reporter gene. For a transfection control 25ng of the *Renilla* luciferase-encoding plasmid pRL-SV40-R*luc* (Promega) was cotransfected. 300ng of pcDNA3.1 MxA, UAP56 or URH49 were used. As a negative control, empty pcDNA3.1 vector was added in the same amount. Cells were lysed 24 hours after transfection and measured using a dual Luciferase assay (Promega).

Western blot and co-immunoprecipitation assays: 293T cells were grown in 10cm cell culture dishes (TPP) and transfected at 80% confluency with the indicated amounts of plasmid. 48 hours after transfection cells were lysed in 500µl lysis buffer (0.5% Triton X-100, 20mM TRIS pH 7.5, 100mM NaCL, 50mM β-glycerolphosphate, 50mM sodiumfluoride, 1mM sodiumorthovanadate and a protease inhibitor cocktail (Roche). Co-immunoprecipitations were perfomed with 1µg mouse anti-FLAG antibodys (SIGMA) for 4 hours at 4°C. Immunoprecipitations were done at room temperature for 1 hour using 50µl

protein-G beads (Dynalbeads, Invitrogen). Samples were washed three times with lysis buffer and beads were taken up in 40µl SDS-Laemmli buffer and heated for 5 minutes at 95°C. Samples were loaded on 10% SDS-gels followed by immunoblot analysis with different antibodies. Anti-FLAG antibody (1:3000, SIGMA) against mouse and rabbit, anti-URH49 (1:750, Acris), anti-UAP56 (serum from a mouse immunized with a peptide of human UAP56), anti-NP mouse monoclonal HB65 (1:3). Primary antibodies were incubated overnight at 4°C and incubated the next day for 1 hour with anti-HRP-conjugated secondary antibodies from GE-Healthcare (1:10000). Membranes were analyzed on a Fuji imager using the Multi Gauge 3.0 software.

Expression constructs and protein purification: URH49 and UAP56 were cloned into pGEX-3x (GE Healthcare) with an N-terminal GST fusion tag (URH49-for: GATAAGAATTCCGCAGAACAGGATGTGGAAAACGATC (EcoRI), URH-49-rev:CTAATGAATTCAATTTACCGGCTCTGCTCGATGTATGTG (EcoRI), UAP56-for: CTTATACCCGGGGCAGAGAACGATGTGGACAATG (SmaI), UAP56-rev: CAATAATCCCGGGATAAACTACCGTGTCTGTTCAATGTA (SmaI). Proteins were expressed in *E.coli* BL21 with 0.1mM IPTG for 4 hours at 32°C. Bacteria were harvested, and lysed by six cycles of sonication for 30 seconds in tris lysis buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 0.1% NP-40, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10% glycerol, protease inhibitor cocktail (Roche)). Lysates were cleared at 12'000g for 20 minutes and applied to a glutathione sepharose column (GE Healthcare Glutathione sepharose High Performance). Bound proteins were washed with tris wash buffer (50mM Tris-HCl pH 8.0, 100mM KCl, 0.1% NP-40, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 10% glycerol), eluted with tris elution buffer (20mM Tris-HCl pH 8.0, 100mM KCl, 0.1% NP-40, 5mM $MgCl_2$, 5mM 2-mercaptoethanol, 10mM reduced glutathione, 20% glycerol) and dialysed against elution buffer without GSH. HIS-MxA and HIS-Mx1 have been previously described [5]. Proteins were expressed in *E.coli* M15 prep4 cells

with 10µM IPTG for 3 hours at 28°C. Bacteria were harvested, and lysed with two french press cycles in tris lysis buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 0.1% NP-40, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 30mM Imidazole, 10% glycerol, protease inhibitor cocktail (Roche)). Lysates were cleared at 20'000g for 20 minutes and applied to a glutathione sepharose column (GE Healthcare Glutathione sepharose High Performance). Bound proteins were washed with tris wash buffer (50mM Tris-HCl pH 8.0, 100mM KCl, 0.1% NP-40, 5mM $MgCl_2$, 10mM 2-mercaptoethanol, 30mM imidazole, 10% glycerol), eluted with tris elution buffer (20mM Tris-HCl pH 8.0, 100mM KCl, 0.1% NP-40, 5mM $MgCl_2$, 5mM 2-mercaptoethanol, 300mM imidazole, 20% glycerol) and dialysed against elution buffer without imidazole.

ALPHA screen: The Alpha screen assays (Perkin Elmer) were performed in a 384-well OptiPlate with 25µl reaction volume. The recombinant proteins were incubated for 2 hours at room temperature at a final concentration of 30nM in Alpha screen buffer (PBS pH 7.2, 0.1% BSA), together with AlphaLISA anti-GST acceptor beads and Alpha screen Ni-chelate donor beads at a concentration of 20µg/ml. Interactions were analyzed using a Perkin Elmer Envision device.

Split GFP system: First, the coding sequences of eGFP aa158-238 including the entire multiple cloning site (MCS) of pEGFP-C1 (Clontech) was amplified by PCR and ligated into the MCS of the pCDNA3.1 (-)neo vector (Invitrogen) yielding the vector pCDNA3.1-GFP158-238. To generate the vector pCDNA3.1 -GFP1-157 the coding sequence of eGFP aa158-238 in the vector pCDNA3.1-GFP158-238 was replaced by a PCR product encoding eGFP aa1-157. Subsequently the open reading frames of human MxA, MxB, UAP56 or URH49 were amplified and introduced in frame at the 3 ' end of the MCS of EGFP. Expression of the split

GFP fusion proteins was verified by western blot analysis using specific antibodies directed against GFP, Mx-protein or UAP56 and URH49.

Indirect immunofluorescence analysis: A549 or 3T3 cells were grown on chamber slides for 24 hours before being transfected. Cells were fixed with 4% paraformaldehyde and mounted in DAPI containing mounting medium (Fluoromount, Southern biotech) when used with the split GFP system. Otherwise cells were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Cells were washed twice with PBS and primary and secondary antibodies were diluted in 1% BSA and incubated for 1 hour at room temperature. Cells were washed three times with PBS after each step. Anti-FLAG antibody (rabbit, 1:2000, Sigma), monoclonal mouse anti-MxA (1:20, Clone 143) were used as primary antibodies. As secondary antibodies, Alexa 488 anti-rabbit and Alexa 594 anti-mouse (1:1000, Invitrogen) were used. Samples were analyzed with a Leica TCS SP5 microscope using the LAF software.

Results

The goal of this study was to assess the interaction of human MxA with the cellular RNA helicases UAP56 and URH49 as well as to study the subcellular localization of MxA *in vivo*. We initially tested whether UAP56 and/or URH49 directly interacted with MxA. For this purpose 293T cells were transfected with plasmids encoding FLAG-tagged UAP56 or URH49 in combination with plasmids encoding MxA. UAP56 and URH49 were immunoprecipitated with an anti-FLAG antibody and the lysates analyzed by western Blot (Fig. 1A). Both helicases, UAP56 as well as URH49, were shown to interact with MxA. To eliminate the possibility of a transfection artefact, we performed co-immunoprecipitations with endogenously expressed proteins and induced MxA expression by treatment with Interferon-α. Human A549 cells were stimulated with Interferon-α overnight to induce MxA expression and immunoprecipitations were performed using a

monoclonal anti-MxA antibody. We analyzed the MxA-associated proteins with a polyclonal antibody detecting both UAP56 and URH49 (SantaCruz Biotechnology). As a control we performed an immunoprecipitaion using a monoclonal antibody against the IAV nucleoprotein (NP). Similar to co-immunoprecipitations with ectopically expressed proteins we demonstrated an interaction between endogenously expressed MxA and UAP56/URH49 (Fig. 1B). To assess the binding affinity we tested the interaction of MxA and UAP56/URH49 *in vitro* using the ALPHA screen technology (Fig 2B). UAP56 and URH49 were expressed with an N-terminal GST-tag and MxA or Mx1 were expressed as HIS-tagged proteins in *E.coli.* The recombinant proteins were affinity purified and used in ALPHA screen assays to evaluate their relative binding strength (Fig. 2A). As a negative control GST only was incubated with MxA or Mx1. To evaluate whether the interaction with UAP56 and URH49 is a common feature of antivirally active Mx proteins we also tested murine Mx1. In contrast to MxA, Mx1 is located in the nucleus [25]. Analysis of the ALPHA screen results revealed a slightly stronger interaction for MxA and UAP56 when compared to the MxA and URH49 interaction. Surprisingly, the interaction of Mx1 with UAP56/URH49 was 2-3 fold stronger as the interaction of MxA with the two helicases (Fig. 2A). Similar to MxA, the interaction between Mx1 and UAP56 appeared to be slightly stronger than between Mx1 and URH49 (Fig. 2C)

Intracellular localization of UAP56/URH49 and MxA

We next wanted to address the question concerning the subcellular localization of the interaction between MxA and UAP56 or URH49. Since MxA is located in the cytoplasm and UAP56 and URH49 in the nucleus [22, 26]. We were interested to find out whether the localization of one of the proteins changed when both proteins were coexpressed [20, 27]. 3T3 cells were transfected with MxA and either FLAG-tagged URH49 or UAP56. As negative controls we expressed each of these proteins alone. Since 3T3 cells express no endogenous

Mx1, we could study the expression of the helicases in absence of endogenous Mx protein (Fig. 3). As expected, UAP56 and URH49 accumulated primarily in the nucleus forming nuclear speckles, as it has been previously described. MxA showed a clear cytoplasmic staining (Fig. 3). Interestingly upon coexpression of MxA with either UAP56 or URH49 we observed accumulation of UAP56 and URH49 in the cytoplasm of cells expressing MxA (Fig. 3) while MxA localization remained the same. UAP56 and URH49 were distributed throughout the whole cell. These results indicate that the described interaction (Fig. 1 and 2) takes place in the cytoplasm.

Split GFP system to characterize the binding of MxA and UAP56/URH49

To verify that the translocation of UAP56 and URH49 is the result of a direct interaction with MxA we constructed a split GFP system to further study the interaction between MxA and UAP56/URH49. For this purpose we adapted a system, which has been previously described [28, 29] fusing the amino acids 1-157 or 158-238 of EGFP to the N-terminus of MxA, UAP56 and URH49, using a 29 aa linker to allow an efficient refolding of the two GFP parts upon interaction of the fusion proteins. Since UAP56 and URH49 have been described to produce homodimers and heterodimers we used this technique to control our UAP56 and URH49 contructs [30] (Fig. 4). For MxA we used its ability to oligomerize as a positive control. We transfected MxA in combination with MxB as a negative control, since no interaction was described for these proteins. In addition, coexpression of the N- and C-terminal half of the GFP protein yielded no fluorescence signal [31]. We observed GFP signal when transfecting GFP1-157-MxA and GFP158-238-MxA, although it was weaker than a full GFP fused to MxA (Fig. 4). As expected, we could see a dimerization of UAP56 or URH49. Upon cotranscfection of MxA and UAP56 or URH49 we observed GFP signal located in the cytoplasm, whereas the homodimers and heterodimer of UAP56

and/or URH49 were located in the nucleus (Fig. 4). These results support our data from the immunofluroescence assays, were similar patterns were observed, revealing a cytoplasmic localization for UAP56 or URH49 upon interaction with MxA.

Dominant-negative effect of UAP56 or URH49 on IAV replication

UAP56 and URH49 have been reported to have a dominant-negative effect on mRNA export if overexpressed [20]. In addition we could recently show that UAP56 is needed for efficient IAV replication as well as for prevention of dsRNA formation upon infection (Wisskirchen et al, submitted). To test whether overexpression of UAP56 or URH49 had an inhibitory effect on IAV replication we used a previously described IAV mini replicon system [32] and compared the results to the known inhibitory activity of MxA on IAV replication [10]. Indeed overexpression of UAP56 and URH49 led to a pronounced inhibition of IAV replication (Fig. 5), confirming our previous results, showing a stronger titer reduction for IAV in cells with a UAP56 knockdown compared to URH49 knockdown cells (Wisskirchen et al, submitted). Surprisingly, the inhibition of replication was slightly stronger than for wildtype MxA, suggesting an effect for UAP56 and URH49 on IAV replication similar to the one described for MxA (Fig. 5).

Discussion

The cellular RNA helicases UAP56 and URH49 play an important role in a variety of cellular processes. UAP56 is required for spliceosome assembly and export of spliced and unspliced mRNA out of the nucleus [16, 18, 20, 22]. Recently, UAP56 was also shown to be important for the nuclear export of certain IAV mRNAs [33]. In addition, UAP56 was shown to bind free viral NP and viral RNPs

[14, 34]. We recently showed that UAP56 is also needed during IAV replication to prevent the accumulation of dsRNA in the cytoplasm of infected cells (Wisskirchen et al), submitted [2, 35]. For IAV and most other Mx-sensitive viruses no direct interaction between Mx-proteins and viral proteins was detected and the molecular mechanism of Mx-proteins against IAV remains elusive. UAP56 might be the missing link between Mx-proteins and IAV. In this study we, show that human MxA and murine Mx1 are indeed able to bind UAP56 and URH49 *in vitro* as well as *in vivo* (Fig. 1, 2). Intriguingly, we observed binding of MxA to UAP56 and URH49 in co-immunoprecipitation experiments despite the fact that MxA is located in the cytoplasm and UAP56/URH49 are predominantly located in the nucleus [20, 24, 27]. Our data clearly show that the interaction of MxA with UAP56/URH49 takes place in the cytoplasm (Fig. 3). Hence, supporting the hypothesis that UAP56 and URH49 are shuttling proteins, this is further supported by our recent observation (Wisskirchen et al, submitted) that knockdown of UAP56 leads to the accumulation of viral dsRNA in the cytoplasm. Previous studies revealed that MxA inhibits a step in IAV replication following primary transcription of mRNA, therefore retention of UAP56 or URH49 in the cytoplasm could be a possible working mechanism [36]. This would lead to an accumulation of UA56/URH49 in the cytoplasm and as a consequence less UAP56 would be available for spliceosome assembly and mRNA export. The primary target for MxA is most likely the mRNA export, since less spliceosome assembly would result in less primary transcription of viral mRNA, which was not shown for MxA. Mx1 exhibits a more efficient antiviral activity against IAV than MxA, which could have two possible explanations (Figure 1, 2) [5, 25, 37]. Either Mx1 binds to UAP56 and URH49 with higher strength than MxA, therefore less UAP56/URH49 would be available for spliceosome assembly and mRNA export, or the reason could be that Mx1 interferes with primary transcription, whereas MxA blocks a later step of IAV replication, most likely in the cytoplasm. Our recent findings about dsRNA accumulation in UAP56 knockdown cells infected with IAV (Wisskirchen et al, submitted) constitute additional evidence for a

cytoplasmic activity of UAP56. Hence, it is conceivable that MxA binds to UAP56 and interfering with the RNA unwinding activity of UAP56, resulting in the observed accumulation of dsRNA. Alternatively, if UAP56 is required in the cytoplasm to redirect viral mRNAs to the ribosomes, this could also be inhibited via a direct binding of MxA and UAP56. To evaluate whether MxA binds directly to UAP56 or URH49 we established a split GFP system. Direct interaction of MxA with UAP56 or URH49 results in the correct association of the N- and C-terminal part of GFP (Fig 4.). Our data clearly show that MxA and UAP56 or URH49 interact in the cytoplasm (Fig. 3, 4). A lot of research has been done using MxA mutants, which lack the ability to form oligomers or the GTPase ability, resulting in proteins, which have no antiviral activity [4, 10]. Using our system will allow us to further analyze if the binding of MxA to UAP56 or URH49 is affected in this mutants or if additional steps are involved in the mechanism of MxA. Since UAP56 is involved in mRNA export and splicing, it might also be interesting to take a closer look, whether MxA affects these cellular processes. Therefore the question rises whether UAP56 is primarily associated with viral RNA. There is growing evidence that the 5` end of viral RNAs exposed to the cytoplasm is associated with the polymerase complex of IAV in order to facilitate translation of viral mRNA [38]. Hence, UAP56 possibly remains associated with the viral polymerase-mRNA complex to facilitate translation via its unwinding activity thereby making it available for MxA. The interaction between IAV NP and URH49 was also confirmed by our previous study, indicating a similar function for UAP56 and URH49 in IAV replication, although UAP56 seems to be more important, since the titer reduction was higher in UAP56 than in URH49 knockdown cells (Wisskirchen et al, submitted). Taken together the interaction of MxA with UAP56 and URH49 could represent a link between the antiviral activity of MxA against IAV, mediating between MxA and viral NP or RNPs.

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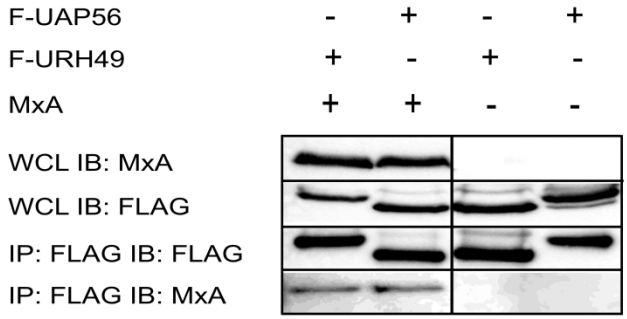
Figures manuscript 2

Figure1

A



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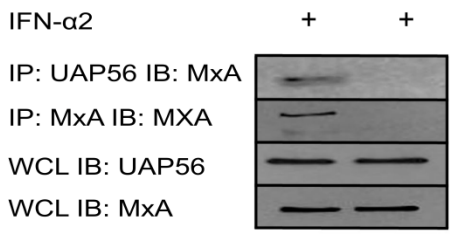
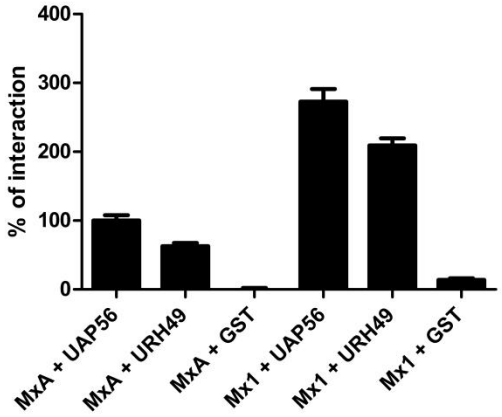


Figure 1: Human MxA interacts with cellular RNA helicases UAP56 and URH49. (A) 293T cells were transfected with FLAG-tagged UAP56 or URH49 together with MxA, cells were lysed after 48 hours and co-immunoprecipitations were performed using anti-FLAG antibodies. (B) Endogenous co-immunoprecipitations were performed using A549 cells. Cells were stimulated overnight with 1000U of Interferon-α, lysed and immunoprecipitations were done using a mouse monoclonal antibody against MxA (left lane), as a negative control a mouse monoclonal antibody against IAV NP was used showing no immunoprecipitaion (right lane). (IP: immunoprecipitation, IB: immunoblot, WCL: whole cell lysate).

Figure 2

A



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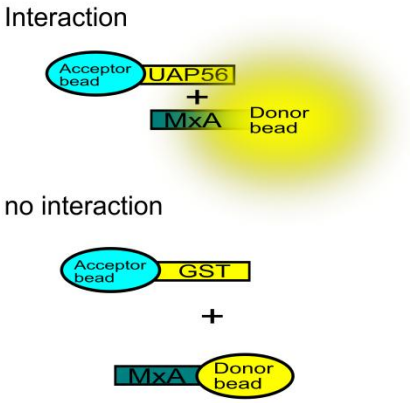

C

	% of interaction
MxA + UAP56	100.00
MxA + URH49	62.95
MxA + GST	0.29
Mx1 + UAP56	272.95
Mx1 + URH49	209.02
Mx1 + GST	13.98

Figure 2: *In vitro* interaction between MxA/Mx1 with UAP56 or URH49. (A) Affinity purified GST-UAP56 or GST-URH49 and HIS-MxA or HIS-Mx1 were mixed and assayed for (30nM of each protein) interaction *in vitro* using the ALPHA screen technology. As a negative control GST alone was incubated with HIS-Mx1 or HIS-MxA. The previously described interaction of UAP56 and MxA was set as a reference for the relative binding strength of the other interactions. (B) Schematics of the ALPHA screen principle. (C) Summary of the relative interactions between the proteins in the ALPHA screen assay.

Figure 3
A

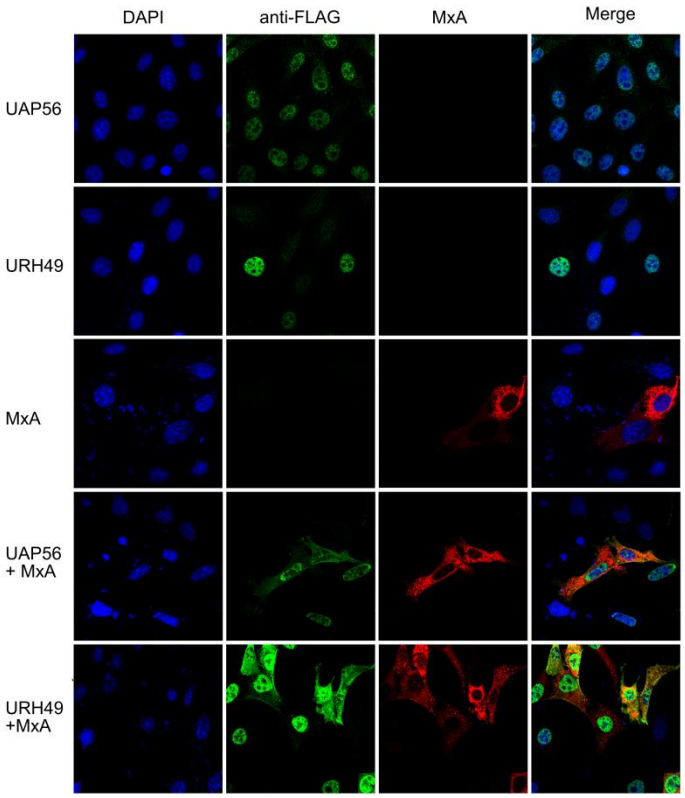

Figure 3: Interaction of MxA and UAP56 or URH49 leads to a shuttling of the helicases into the cytoplasm. (A) 3T3 cells were transfected with FLAG-tagged UAP56 or URH49 with or without MxA. Cells were fixed with 4% formaldehyde and permeabilzed with 0.5% Triton X-100. Cells were stained using a polyclonal anti-FLAG antibody (1:2000) and anti-MxA antibody (1:20. Clone143). Pictures were taken with a Leica TCS-SP5 confocal microscope.

Figure 4
A

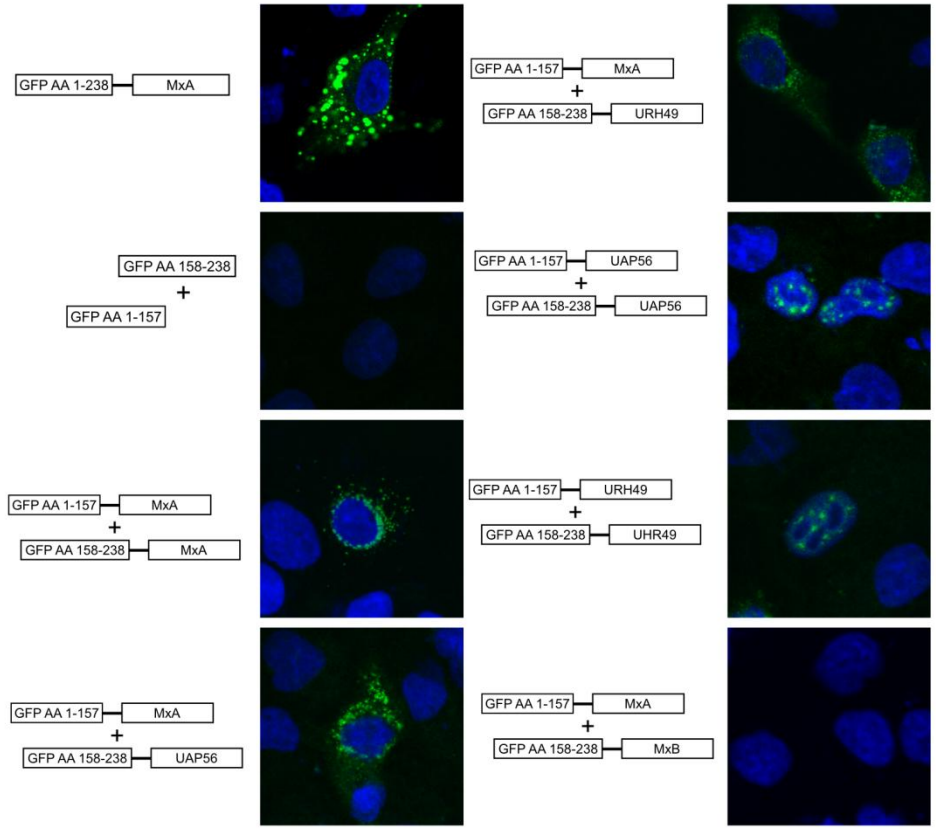

Figure 4: Split GFP system using MxA and UAP56 or URH49 constructs. (A) A549 cells were transfected with the indicated constructs, resulting in GFP signal upon protein-protein interaction. As a negative control MxA and MxB were used as well as the corresponding split GFPs without a protein fused to the C-terminus. Pictures were taken with a Leica TCS-SP5 confocal microscope.

Figure 5

A

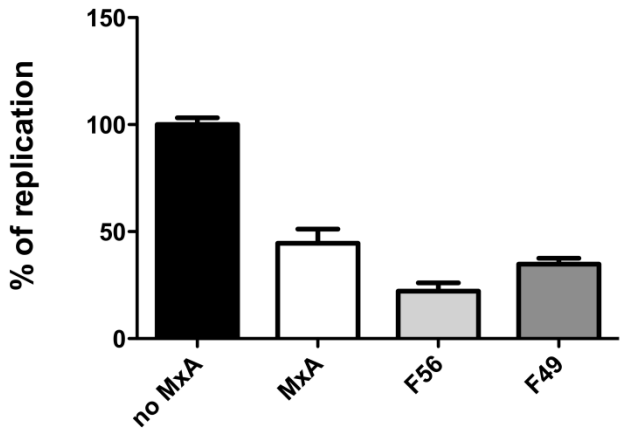

Figure 5: Dominant-negative effect of UAP56 and URH49 in an IAV mini replicon system. (A) 293T cells were transfected using an IAV mini replicon system derived from A/Thailand/(KAN-1)/2004. The empty vector control was set as a reference. Signals were measured using a dual luciferase reporter assay (Promega).

References

1. Haller, O., S. Stertz, and G. Kochs, *The Mx GTPase family of interferon-induced antiviral proteins.* Microbes Infect, 2007. **9**(14-15): p. 1636-43.
2. Horisberger, M.A., *Interferon-induced human protein MxA is a GTPase which binds transiently to cellular proteins.* J Virol, 1992. **66**(8): p. 4705-9.
3. Warnock, D.E., J.E. Hinshaw, and S.L. Schmid, *Dynamin self-assembly stimulates its GTPase activity.* J Biol Chem, 1996. **271**(37): p. 22310-4.
4. Di Paolo, C., et al., *Intramolecular backfolding of the carboxyl-terminal end of MxA protein is a prerequisite for its oligomerization.* J Biol Chem, 1999. **274**(45): p. 32071-8.
5. Pitossi, F., et al., *A functional GTP-binding motif is necessary for antiviral activity of Mx proteins.* J Virol, 1993. **67**(11): p. 6726-32.
6. Schwemmle, M., et al., *Vesicular stomatitis virus transcription inhibited by purified MxA protein.* Virology, 1995. **206**(1): p. 545-54.
7. Pavlovic, J., et al., *Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein.* J Virol, 1990. **64**(7): p. 3370-5.
8. Landis, H., et al., *Human MxA protein confers resistance to Semliki Forest virus and inhibits the amplification of a Semliki Forest virus-based replicon in the absence of viral structural proteins.* J Virol, 1998. **72**(2): p. 1516-22.
9. Schnorr, J.J., et al., *MxA-dependent inhibition of measles virus glycoprotein synthesis in a stably transfected human monocytic cell line.* J Virol, 1993. **67**(8): p. 4760-8.
10. Gao, S., et al., *Structural basis of oligomerization in the stalk region of dynamin-like MxA.* Nature, 2010. **465**(7297): p. 502-6.
11. Kochs, G. and O. Haller, *Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2082-6.
12. Kochs, G., et al., *Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 3153-8.
13. Accola, M.A., et al., *The antiviral dynamin family member, MxA, tubulates lipids and localizes to the smooth endoplasmic reticulum.* J Biol Chem, 2002. **277**(24): p. 21829-35.
14. Momose, F., et al., *Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis.* J Virol, 2001. **75**(4): p. 1899-908.
15. Fleckner, J., et al., *U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction.* Genes Dev, 1997. **11**(14): p. 1864-72.
16. Shen, H., *UAP56- a key player with surprisingly diverse roles in pre-mRNA splicing and nuclear export.* BMB Rep, 2009. **42**(4): p. 185-8.

17. Shen, H., et al., *Distinct activities of the DExD/H-box splicing factor hUAP56 facilitate stepwise assembly of the spliceosome.* Genes Dev, 2008. **22**(13): p. 1796-803.
18. Luo, M.L., et al., *Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly.* Nature, 2001. **413**(6856): p. 644-7.
19. Carmody, S.R. and S.R. Wente, *mRNA nuclear export at a glance.* J Cell Sci, 2009. **122**(Pt 12): p. 1933-7.
20. Kota, K.P., et al., *Binding of ATP to UAP56 is necessary for mRNA export.* J Cell Sci, 2008. **121**(Pt 9): p. 1526-37.
21. Pryor, A., et al., *Growth-regulated expression and G0-specific turnover of the mRNA that encodes URH49, a mammalian DExH/D box protein that is highly related to the mRNA export protein UAP56.* Nucleic Acids Res, 2004. **32**(6): p. 1857-65.
22. Kapadia, F., et al., *Nuclear localization of poly(A)+ mRNA following siRNA reduction of expression of the mammalian RNA helicases UAP56 and URH49.* Gene, 2006. **384**: p. 37-44.
23. Meignin, C. and I. Davis, *UAP56 RNA helicase is required for axis specification and cytoplasmic mRNA localization in Drosophila.* Dev Biol, 2008. **315**(1): p. 89-98.
24. Schmidt, U., et al., *Assembly and mobility of exon-exon junction complexes in living cells.* RNA, 2009. **15**(5): p. 862-76.
25. Zurcher, T., J. Pavlovic, and P. Staeheli, *Nuclear localization of mouse Mx1 protein is necessary for inhibition of influenza virus.* J Virol, 1992. **66**(8): p. 5059-66.
26. Aebi, M., et al., *cDNA structures and regulation of two interferon-induced human Mx proteins.* Mol Cell Biol, 1989. **9**(11): p. 5062-72.
27. Haller, O. and G. Kochs, *Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity.* Traffic, 2002. **3**(10): p. 710-7.
28. Ghosh, I., A.D. Hamilton, and L. Regan, *Antiparallel leucine zipper-directed protein reassembly: Application to the green fluorescent protein.* Journal of the American Chemical Society, 2000. **122**(23): p. 5658-5659.
29. Hu, C.D., Y. Chinenov, and T.K. Kerppola, *Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation.* Mol Cell, 2002. **9**(4): p. 789-98.
30. Zhao, R., et al., *Crystal structure of UAP56, a DExD/H-box protein involved in pre-mRNA splicing and mRNA export.* Structure, 2004. **12**(8): p. 1373-81.
31. Melen, K. and I. Julkunen, *Nuclear cotransport mechanism of cytoplasmic human MxB protein.* J Biol Chem, 1997. **272**(51): p. 32353-9.
32. Maenz, B., et al., *Disruption of the viral polymerase complex assembly as a novel approach to attenuate influenza A virus.* J Biol Chem, 2011.
33. Read, E.K. and P. Digard, *Individual influenza A virus mRNAs show differential dependence on cellular NXF1/TAP for their nuclear export.* J Gen Virol, 2010. **91**(Pt 5): p. 1290-301.

34. Mayer, D., et al., *Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches.* J Proteome Res, 2007. **6**(2): p. 672-82.
35. Engelhardt, O.G., et al., *Interferon-induced antiviral Mx1 GTPase is associated with components of the SUMO-1 system and promyelocytic leukemia protein nuclear bodies.* Exp Cell Res, 2001. **271**(2): p. 286-95.
36. Pavlovic, J., O. Haller, and P. Staeheli, *Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle.* J Virol, 1992. **66**(4): p. 2564-9.
37. Zurcher, T., J. Pavlovic, and P. Staeheli, *Mechanism of human MxA protein action: variants with changed antiviral properties.* EMBO J, 1992. **11**(4): p. 1657-61.
38. Burgui, I., et al., *Influenza virus mRNA translation revisited: is the eIF4E cap-binding factor required for viral mRNA translation?* J Virol, 2007. **81**(22): p. 12427-38.

Discussion

The goal of this thesis was to address the question how the antiviral active proteins of the Mx-family affect the IAV replication and exert their antiviral potential. Despite numerous efforts by several research groups no evidence is known for a direct interaction of MxA with the viral NPs or RNPs leading us to hypothesize that Mx-proteins inhibit IAV replication via cellular factors. It was therefore conceivable to test cellular factors playing a role in IAV replication, which lead us to a detailed analysis of UAP56 and URH49 as possible candidates.

Role of UAP56 and URH49

UAP56 is a cellular mRNA helicase with several functions, including mRNA export and the involvement in multiple steps for spliceosome assembly and activity. UAP56 can recruit cellular factors including ALY/REF or CIP29 to the nascent mRNA and promotes nuclear export [50, 81, 82]. URH49 shares 90% identity at the amino acid level with UAP56 and was shown to have similar functions, though less is known about the exact role of URH49 [83]. UAP56 has been shown to stimulate IAV RNA synthesis *in vitro*, as well as to bind to IAV NP. UAP56 was also shown to be important for nuclear export of a set of IAV mRNAs, whereas not all viral mRNAs were dependent on UAP56, nothing is known for URH49 regarding IAV so far [36, 58, 79]. In this study we show that UAP56 and URH49 are important for IAV replication, since we observed a titer reduction in knockdown cells for UAP56 and URH49 (Manuscript 1, Figure 2). A set of different Influenza strains was used to infect knockdown cells and a 10-fold titer reduction was observed for UAP56 and a 5-fold reduction for URH49 using the human laboratory strain A/34/PR8/(H1N1) or a recent pandemic isolate

A/Zurich/8665/2009/(H1N1) (Manuscript 1, Figure 2 C, D). We showed that UAP56 appears to be much more important for the replication of avian IAV, resulting in a 200-fold titer reduction, whereas only a 5-fold reduction could be seen for URH49, suggesting that UAP56 plays an important role in avian IAV replication (Manuscript 1, Figure 2B). To test whether UAP56 and URH49 affect splicing or mRNA export of IAV we isolated cytoplasmic or nuclear RNA of infected cells and analyzed the splicing pattern of the M-segment as well as the export of NP, M1, HA and NS1 mRNAs via qPCR and or northern blot (Manuscript 1, Figure 3). Like previously published, UAP56 seems to affect the mRNA export of M1 mRNA, whereas a minor effect could be seen for HA and NS1 mRNA [36]. A similar effect was observed for URH49, but surprisingly the export of mRNAs was more strongly inhibited than in UAP56-knockdown cells (Manuscript 1, Figure 3), despite the fact that in URH49-knockdown cells viral titers were only slightly reduced.

UAP56 prevents dsRNA accumulation

Since the nuclear export phenotype did not appear to have a huge impact on viral titers, we next examined whether the unwinding activity of UAP56 and URH49 play a role. IAV is lacking a virally encoded helicase and therefore, it may use a cellular RNA helicase for efficient replication. Because UAP56 and URH49 are both RNA helicases with a known dsRNA unwinding activity we wanted to address the question whether one or both helicases are involved in preventing the formation of dsRNA intermediates during IAV infection. IAV is known not to produce dsRNA intermediates, indicating an involvement of cellular RNA helicases [84]. To analyze whether dsRNA plays a role we first tested for a PKR-activation in UAP56- or URH49-siRNA knockdown cells. We observed a slight background of P-PKR in control siRNA cells infected with virus, but a stronger activation in knockdown cells infected with either FPV or PR8. Although we could also see P-PKR in mock infected UAP56 or URH49 knockdown cells

this is most likely because the helicases might be involved in the unwinding of cellular RNAs (Manuscript 1, Figure 4). Furthermore we tried to detect dsRNA using an immunofluroescence-based approach with an antibody specific for dsRNA. UAP56- or URH49-knockdown cells were infected with FPV or PR8 virus. Infection with FPV and PR8 lead to an accumulation of dsRNA in UAP56 knockdown cells whereas no dsRNA was observed in URH49 knockdown cells (Manuscript 1, Figure 5 A, B). A possible explanation might be that UAP56 is able to fully complement the function of URH49 but URH49 can complement UAP56 only partially. To test whether the dsRNA was of viral origin we used actinomycin D to completely block IAV transcription or cycloheximide only allowing primary transcription but no amplification of viral RNA. Both inhibitors prevented the formation of dsRNA, indirectly ruling out that the observed dsRNA is of cellular mRNA origin, which should be still detectable otherwise (Manuscript 1, Figure 5 A, B). In addition leptomycin B which is described to inhibit the CRM-1 dependent export of IAV RNPs [36, 38] had no effect on the dsRNA formation (data not shown), supporting our hypothesis of a viral mRNA versus vRNPs. As an additional control we used VSV to exert a more general effect of UAP56 or URH49 on negative stranded RNA viruses. In UAP56 knockdown cells a 10-fold titer reduction was observed, whereas only a 4-fold reduction was observed for URH49 (Manuscript 1, Figure 2 E). Furthermore, we also detected dsRNA accumulation in VSV infected UAP56 knockdown cells, this was not the case for URH49, further supporting our observations for IAV. This makes UAP56 potentially important for prevention of dsRNA formation during infection an additional virus, leading to an eventually more general mechanism (Manuscript 1, Figure 7).

MxA interacts with UAP56 and URH49

Our initial findings, of both helicases interacting with human and avian IAV NPs (Manuscript 2, Figure 1 A, B) were made when we also tested whether the human MxA protein interacted with UAP56 or URH49. So far several groups have described the antiviral function of MxA, still leaving the mechanism unclear, since no direct interaction with any viral IAV protein was shown [65, 67, 75, 76, 85]. We were able to show an interaction between exogenous as well as endogenous UAP56 or URH49 and human MxA (Manuscript 2, Figure 1 A, B). In addition, we performed *in vitro* ALPHA screen assays to analyze the binding of both helicases with MxA and Mx1 the murine member of the Mx-family. Mx1 accumulates in the nucleus, in contrast to MxA. We observed a stronger relative binding of Mx1 to UAP56 or URH49 compared to MxA (Manuscript 2, Figure 2). We observed a similar correlation for the relative strength of the interaction comparing UAP56 and URH49, since the interaction with UAP56 and MxA or Mx1 was slightly stronger compared to URH49 and MxA or Mx1. Furthermore, we wanted to address the question in which cellular compartment the interaction between UAP56 and MxA takes place, since MxA is located in the cytoplasm and UAP56 and URH49 are both located in the nucleus forming characteristic nuclear speckles [65, 81]. Although MxA mutants with an NLS attached to their N-terminus are re-located to the nucleus have an even higher antiviral activity compared to wild type MxA. No shuttling of MxA has so been described so far or any interaction with nuclear proteins [76]. Cotransfection of MxA and UAP56 or URH49 in 3T3 cells lacking endogenous Mx1 resulted in a translocation of UAP56 and URH49 into the cytoplasm of the cells, whereas in control cells lacking MxA expression both UAP56 and URH49 remained confined to the nucleus. Although UAP56 has been previously hypothesized to play a role in mRNA translocation in the cytoplasm in redirecting mRNAs to the translation machinery, these findings give new insights into the role of both helicases (Manuscript 2, Figure 3) [86]. To verify a direct interaction between MxA and UAP56 or URH49 we established a split GFP system for MxA and the two

helicases. The GFP signal was complemented when a direct interaction of MxA with UAP56 or URH49 occurred. We used MxA and MxB as negative controls, as well as only the GFP parts, resulting in no signal. We observed a strong signal for the MxA/MxA interaction which is the result of the oligomerization of MxA, we also observed GFP signal for the homodimeric interactions of UAP56 and URH49 as well as for the MxA-UAP56 and MxA-URH49 interaction, supporting our previous findings about this interaction (Manuscript 2, Figure 4). Although the homodimer of UAP56 and URH49 was present in the nucleus of the cell, the interaction of MxA-UAP56 and MxA-URH49 occurs in the cytoplasm of the cell, indicating that MxA is capable of retaining the two shuttling helicases in the cytoplasm. These results confirm our observations from the cotranscfection experiments.

Dominate-negative effect of UAP56 or URH49 on IAV replication

To demonstrate a link between IAV replication, UAP56/URH49 and the Mx-proteins we then analyzed whether the described dominant-negative effect of UAP56 also plays a role in the context of an artificial mini replicon system of IAV. Cotransfection of the mini replicon system with MxA was used as a positive control. Surprisingly, we observed an even stronger reduction when UAP56 and URH49 were overexpressed (Manuscript 2, Figure 5). Taken together, we can demonstrate a pivotal role for UAP56 in IAV replication, as well as a direct interaction between MxA and UAP56 and URH49, giving us the opportunity to get more insights into the molecular mechanism of the function of MxA. So far, URH49 has been only described in mammals [48], whereas birds only express UAP56. This fact could be important, since we observed a 200-fold reduction of viral titer for avian IAV in UAP56 knockdown cells (Manuscript 1, Figure 2 B). We think this is due to a lack of compensation by URH49 since birds lack this gene. Therefore, the virus might not be able to utilize it as efficiently as human influenza viruses in case of a UAP56 knockdown. Since humans express UAP56

and URH49, the virus could adapt to the situation and use the available helicases for efficient replication. Nevertheless, UAP56 seems to play a more important role since it also prevents IAV from accumulating dsRNA in the cytoplasm of infected cells, whereas this was not the case in URH49 knockdown cells. We think that the prevention of dsRNA accumulation is one of the roles of UAP56, which his very plausible since IAV is believed to recruit cellular helicase and is known to be dependent on the cellular mRNA export machinery, thus making UAP56 an optimal candidate for this purpose.

Working hypothesis for UAP56 and MxA

In addition our data obtained with VSV revealed a more general role of UAP56 for negative-stranded RNA virus replication, although there are major differences between VSV and IAV. Most importantly, VSV replicates in the cytoplasm of infected cells, whereas IAV replicates in the nucleus. Nevertheless, it would be interesting to test a VSV mutant that was described to have a single amino acid mutation in the N-protein resulting in production of large amounts of dsRNA, if this can be correlated with UAP56 or URH49 binding or utilization [87]. For VSV additional effects might be possible, since the virus is not as dependent on the cellular export machinery as IAV. Hence, it could have additional implications on cellular processes that are beneficial for an enhanced viral production, as long as they do not trigger any viral countermeasures, e.g. the type I interferon system. Since our initial focus was on MxA and a further elucidation of the molecular mechanism, the interactions of MxA and Mx1 with the two helicases could give us further insight into how this might work. Although UAP56 and URH49 are located in the nucleus of the cell, whereas MxA accumulates in the cytoplasm, we could show a retention of the two helicases into the cytoplasm by MxA. This strongly suggests that UAP56 and URH49 are shuttling proteins. To proof a more general involvement of UAP56 and URH49 we also tested Mx1 for an interaction with the two helicases, which was not surprisingly even higher than for MxA

(Manuscript 2). UAP56 and URH49 have been described to be important for the export of intronless mRNAs for human cytomegalovirus (HCMV), leading to replication deficient viruses when the viral pUL69 protein was mutated to be unable to bind UAP56 [60]. This data suggests a role for UAP56 and URH49 in different virus replication cycles and further underlines the importance of nuclear-cytoplasmic shuttling of UAP56 and URH49 [61]. For UAP56 there is evidence that it might be important for redirecting mRNAs to their final destination in the cytoplasm, however this has so far only been shown in oocytes and not in human cells [86]. We could show that UAP56 and URH49 shuttle between nucleus and cytoplasm therefore making it very likely for them to have a function in the cytoplasm as well, which could be used either by the cellular mRNA export machinery, viruses or antiviral proteins. Since UAP56 is able to bind MxA and IAV NPs the results of our two studies can be combined to form a hypothesis how MxA interferes with IAV replication. Mx1 has been shown to inhibit primary transcription of IAV, still the exact mechanism remains unknown [75]. A possible explanation might be that Mx1 binds to UAP56 and or URH49 and therefore inhibits mature spliceosome assembly, which would result in an inhibition of viral and cellular transcription. This could further be supported by the model of Mx-protein oligomerization since data is available suggesting that the oligomeric form of Mx proteins is only a storage form, undergoing conformational changes to become antivirally active. Possibly MxA is antivirally active as a monomer but also becomes toxic for the cell itself [65, 88, 89]. Although this might be the fact for Mx1, we cannot assume this for MxA, since MxA i) neither inhibits primary transcription of IAV but rather affects a later step in replication, ii) nor is there any evidence for nuclear-cytoplasmic shuttling of MxA. It is highly unlikely that MxA complexes passively diffuse through the nuclear pore complex since they are too large for this possibility, even if they would be actively imported the large size of the oligomers would prevent nuclear import. The interaction between NP and UAP56 can occur in the nucleus of the infected cell, whereas the interaction between UAP56 and MxA can only take place in the cytoplasm. It is therefore

possible that UAP56 is the common denominator between IAV NP and MxA, eventually allowing MxA to recruit UAP56 and/or URH49 for the antiviral activity, which would have several effects. MxA could make use of the nuclear-cytoplasmic shuttling from UAP56 and URH49. A possible explanation is that MxA actively sequesters UAP56 and URH49 that shuttle out of the nucleus, leading to a decrease of the RNA helicases in the nucleus (**Figure 8**). Since it is known that UAP56 can stimulate viral RNA synthesis *in vitro* a lack of the helicase would result in less cRNA or vRNA, whereas fewer UAP56 available for mature spliceosome assembly would result in a lesser amount of viral mRNAs, in both cases this would lead to a less efficient viral replication [58].

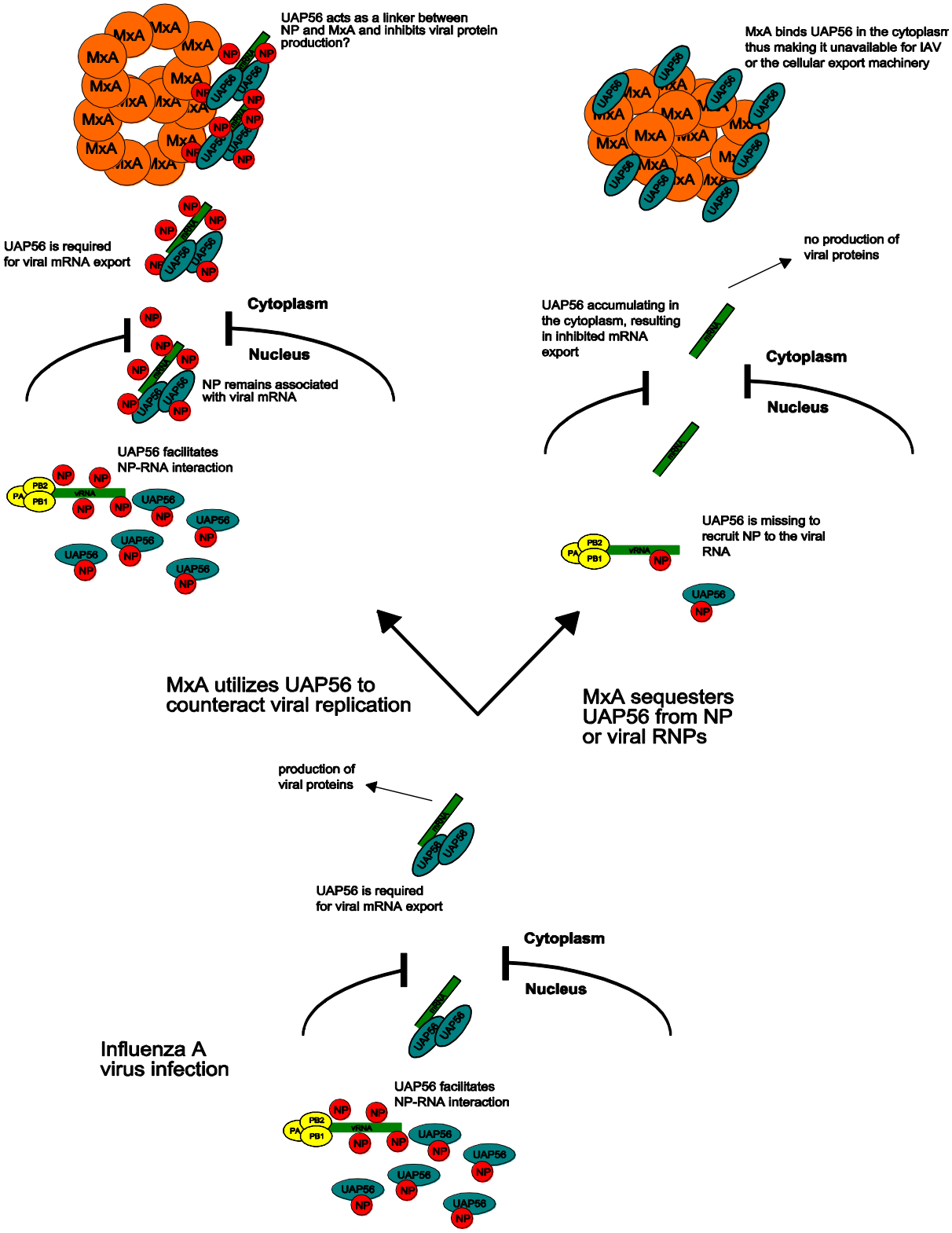

Figure 8: Working hypothesis of UAP56 and MxA

Taken together, we come up with two possibilities for the importance of the UAP56-MxA interaction to play a role in the mechanism of MxA. i) The interaction of MxA and UAP56 leads to a sequestration of the helicase therefore inhibiting an efficient viral mRNA export or maybe even the mRNA transport to the ribosomes. This is further supported by the findings of Read et *al*. and our own data (Manuscript 1), showing a nuclear retention of certain viral mRNA upon infection in UAP56 knockdown cells [36]. ii) The MxA-UAP56 interaction inhibits the helicase activity of UAP56, which could be further supported by our findings, that dsRNA accumulates in UAP56 knockdown cells infected with IAV (Manuscript 1, Figure 5). Both are possible scenarios and we will further investigate our hypotheses to gain further insights regarding a possible molecular mechanism of MxA. In summary, we could describe an interaction between MxA and the two cellular helicases UAP56 and URH49, which was also shown for the murine homologue Mx1. In addition, we showed that UAP56 plays a pivotal role in IAV replication, preventing the formation of dsRNA during infection. Thus, we could describe for the first time a direct interaction of MxA with two nuclear RNA helicases, as well as the involvement of two cellular helicases for IAV replication. UAP56 appears to be the cellular factor bridging an interaction between MxA and IAV NP and we will try to investigate this more in detail in the future.

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References

1. Lemon, S.M. and A.A. Mahmoud, *The threat of pandemic influenza: are we ready?* Biosecur Bioterror, 2005. **3**(1): p. 70-3.
2. Fouchier, R.A., et al., *Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls.* J Virol, 2005. **79**(5): p. 2814-22.
3. Johnson, N.P. and J. Mueller, *Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic.* Bull Hist Med, 2002. **76**(1): p. 105-15.
4. Garten, R.J., et al., *Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans.* Science, 2009. **325**(5937): p. 197-201.
5. Fields, B.N., D.M. Knipe, and P.M. Howley, *Fields virology*. 4th ed. / editors-in-chief, David M. Knipe, Peter M. Howley, associate editors, Diane E. Griffin ... [et al.] ed2001, Philadelphia ; London: Lippincott Williams & Wilkins. 2 v.
6. Flick, R. and G. Hobom, *Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation.* J Gen Virol, 1999. **80 (Pt 10)**: p. 2565-72.
7. Skehel, J.J. and D.C. Wiley, *Influenza haemagglutinin.* Vaccine, 2002. **20 Suppl 2**: p. S51-4.
8. Bullough, P.A., et al., *Structure of influenza haemagglutinin at the pH of membrane fusion.* Nature, 1994. **371**(6492): p. 37-43.
9. Skehel, J.J. and D.C. Wiley, *Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin.* Annu Rev Biochem, 2000. **69**: p. 531-69.
10. Wilson, G.S., et al., *Topley & Wilson's microbiology & microbial infections. Virology*. 10th ed2005, London: Hodder Arnold.
11. Ludwig, S., S. Pleschka, and T. Wolff, *A fatal relationship--influenza virus interactions with the host cell.* Viral Immunol, 1999. **12**(3): p. 175-96.
12. Leiding, T., et al., *Proton and cation transport activity of the M2 proton channel from influenza A virus.* Proc Natl Acad Sci U S A, 2010. **107**(35): p. 15409-14.
13. Pinto, L.H. and R.A. Lamb, *The M2 proton channels of influenza A and B viruses.* J Biol Chem, 2006. **281**(14): p. 8997-9000.
14. Portela, A. and P. Digard, *The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication.* J Gen Virol, 2002. **83**(Pt 4): p. 723-34.

15. Huang, X., et al., *Effect of influenza virus matrix protein and viral RNA on ribonucleoprotein formation and nuclear export.* Virology, 2001. **287**(2): p. 405-16.
16. Beaton, A.R. and R.M. Krug, *Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end.* Proc Natl Acad Sci U S A, 1986. **83**(17): p. 6282-6.
17. Coloma, R., et al., *The structure of a biologically active influenza virus ribonucleoprotein complex.* PLoS Pathog, 2009. **5**(6): p. e1000491.
18. Jorba, N., R. Coloma, and J. Ortin, *Genetic trans-complementation establishes a new model for influenza virus RNA transcription and replication.* PLoS Pathog, 2009. **5**(5): p. e1000462.
19. Bui, M., G. Whittaker, and A. Helenius, *Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins.* J Virol, 1996. **70**(12): p. 8391-401.
20. Bui, M., et al., *Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins.* J Virol, 2000. **74**(4): p. 1781-6.
21. Martin, K. and A. Helenius, *Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import.* Cell, 1991. **67**(1): p. 117-30.
22. Garcia-Sastre, A., et al., *Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems.* Virology, 1998. **252**(2): p. 324-30.
23. Garcia-Sastre, A., *Antiviral response in pandemic influenza viruses.* Emerg Infect Dis, 2006. **12**(1): p. 44-7.
24. Bergmann, M., et al., *Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication.* Journal of Virology, 2000. **74**(13): p. 6203-6206.
25. Talon, J., et al., *Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein.* J Virol, 2000. **74**(17): p. 7989-96.
26. Wang, X., et al., *Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon.* J Virol, 2000. **74**(24): p. 11566-73.
27. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates.* Science, 2006. **314**(5801): p. 997-1001.
28. Rehwinkel, J., et al., *RIG-I detects viral genomic RNA during negative-strand RNA virus infection.* Cell, 2010. **140**(3): p. 397-408.
29. Zhirnov, O.P., et al., *NS1 protein of influenza A virus down-regulates apoptosis.* J Virol, 2002. **76**(4): p. 1617-25.
30. Neumann, G., M.T. Hughes, and Y. Kawaoka, *Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1.* EMBO J, 2000. **19**(24): p. 6751-8.
31. Nayak, D.P., E.K. Hui, and S. Barman, *Assembly and budding of influenza virus.* Virus Res, 2004. **106**(2): p. 147-65.
32. Kogure, T., et al., *Human trachea primary epithelial cells express both sialyl(alpha2-3)Gal receptor for human parainfluenza virus type 1 and*

avian influenza viruses, and sialyl(alpha2-6)Gal receptor for human influenza viruses. Glycoconj J, 2006. **23**(1-2): p. 101-6.
33. Kemler, I., G. Whittaker, and A. Helenius, *Nuclear import of microinjected influenza virus ribonucleoproteins.* Virology, 1994. **202**(2): p. 1028-33.
34. Mark, G.E., et al., *Nuclear Accumulation of Influenza Viral-Rna Transcripts and the Effects of Cycloheximide, Actinomycin-D, and Alpha-Amanitin.* Journal of Virology, 1979. **29**(2): p. 744-752.
35. Dias, A., et al., *The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit.* Nature, 2009. **458**(7240): p. 914-8.
36. Read, E.K. and P. Digard, *Individual influenza A virus mRNAs show differential dependence on cellular NXF1/TAP for their nuclear export.* J Gen Virol, 2010. **91**(Pt 5): p. 1290-301.
37. Doms, R.W., *Protein conformational changes in virus-cell fusion.* Methods Enzymol, 1993. **221**: p. 61-72.
38. Elton, D., et al., *Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway.* J Virol, 2001. **75**(1): p. 408-19.
39. Bush, R.M., et al., *Positive selection on the H3 hemagglutinin gene of human influenza virus A.* Mol Biol Evol, 1999. **16**(11): p. 1457-65.
40. Plotkin, J.B. and J. Dushoff, *Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 7152-7.
41. Itoh, Y., et al., *In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses.* Nature, 2009. **460**(7258): p. 1021-5.
42. Fuller-Pace, F.V., *DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation.* Nucleic Acids Res, 2006. **34**(15): p. 4206-15.
43. Tanner, N.K. and P. Linder, *DExD/H box RNA helicases: from generic motors to specific dissociation functions.* Mol Cell, 2001. **8**(2): p. 251-62.
44. Caruthers, J.M. and D.B. McKay, *Helicase structure and mechanism.* Curr Opin Struct Biol, 2002. **12**(1): p. 123-33.
45. Silverman, E., G. Edwalds-Gilbert, and R.J. Lin, *DExD/H-box proteins and their partners: helping RNA helicases unwind.* Gene, 2003. **312**: p. 1-16.
46. Cordin, O., et al., *The DEAD-box protein family of RNA helicases.* Gene, 2006. **367**: p. 17-37.
47. Fleckner, J., et al., *U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction.* Genes Dev, 1997. **11**(14): p. 1864-72.
48. Pryor, A., et al., *Growth-regulated expression and G0-specific turnover of the mRNA that encodes URH49, a mammalian DExH/D box protein that is highly related to the mRNA export protein UAP56.* Nucleic Acids Res, 2004. **32**(6): p. 1857-65.
49. Kapadia, F., et al., *Nuclear localization of poly(A)+ mRNA following siRNA reduction of expression of the mammalian RNA helicases UAP56 and URH49.* Gene, 2006. **384**: p. 37-44.

50. Luo, M.L., et al., *Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly.* Nature, 2001. **413**(6856): p. 644-7.
51. Zhao, R., et al., *Crystal structure of UAP56, a DExD/H-box protein involved in pre-mRNA splicing and mRNA export.* Structure, 2004. **12**(8): p. 1373-81.
52. Shi, H., et al., *Crystal structure of the human ATP-dependent splicing and export factor UAP56.* Proc Natl Acad Sci U S A, 2004. **101**(51): p. 17628-33.
53. Shen, J., L. Zhang, and R. Zhao, *Biochemical characterization of the ATPase and helicase activity of UAP56, an essential pre-mRNA splicing and mRNA export factor.* J Biol Chem, 2007. **282**(31): p. 22544-50.
54. Hastings, M.L. and A.R. Krainer, *Pre-mRNA splicing in the new millennium.* Curr Opin Cell Biol, 2001. **13**(3): p. 302-9.
55. Strasser, K. and E. Hurt, *Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p.* Nature, 2001. **413**(6856): p. 648-52.
56. Stutz, F., et al., *REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export.* RNA, 2000. **6**(4): p. 638-50.
57. Kiesler, E., F. Miralles, and N. Visa, *HEL/UAP56 binds cotranscriptionally to the Balbiani ring pre-mRNA in an intron-independent manner and accompanies the BR mRNP to the nuclear pore.* Curr Biol, 2002. **12**(10): p. 859-62.
58. Momose, F., et al., *Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis.* J Virol, 2001. **75**(4): p. 1899-908.
59. Lischka, P., et al., *The UL69 transactivator protein of human cytomegalovirus interacts with DEXD/H-Box RNA helicase UAP56 to promote cytoplasmic accumulation of unspliced RNA.* Mol Cell Biol, 2006. **26**(5): p. 1631-43.
60. Zielke, B., et al., *Characterization of the Betaherpesviral pUL69 Protein Family Reveals Binding of the Cellular mRNA Export Factor UAP56 as a Prerequisite for Stimulation of Nuclear mRNA Export and for Efficient Viral Replication.* J Virol, 2011. **85**(4): p. 1804-19.
61. Toth, Z., P. Lischka, and T. Stamminger, *RNA-binding of the human cytomegalovirus transactivator protein UL69, mediated by arginine-rich motifs, is not required for nuclear export of unspliced RNA.* Nucleic Acids Res, 2006. **34**(4): p. 1237-49.
62. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
63. Samuel, C.E., *Antiviral actions of interferons.* Clin Microbiol Rev, 2001. **14**(4): p. 778-809, table of contents.
64. Lindenmann, J., *Resistance of mice to mouse-adapted influenza A virus.* Virology, 1962. **16**: p. 203-4.

65. Haller, O. and G. Kochs, *Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity.* Traffic, 2002. **3**(10): p. 710-7.
66. Pavlovic, J., et al., *Enhanced virus resistance of transgenic mice expressing the human MxA protein.* J Virol, 1995. **69**(7): p. 4506-10.
67. Pavlovic, J., et al., *Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein.* J Virol, 1990. **64**(7): p. 3370-5.
68. Accola, M.A., et al., *The antiviral dynamin family member, MxA, tubulates lipids and localizes to the smooth endoplasmic reticulum.* J Biol Chem, 2002. **277**(24): p. 21829-35.
69. Pitossi, F., et al., *A functional GTP-binding motif is necessary for antiviral activity of Mx proteins.* J Virol, 1993. **67**(11): p. 6726-32.
70. Gao, S., et al., *Structural basis of oligomerization in the stalk region of dynamin-like MxA.* Nature, 2010. **465**(7297): p. 502-6.
71. Kochs, G. and O. Haller, *Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2082-6.
72. Reichelt, M., et al., *Missorting of LaCrosse virus nucleocapsid protein by the interferon-induced MxA GTPase involves smooth ER membranes.* Traffic, 2004. **5**(10): p. 772-84.
73. Kochs, G. and O. Haller, *GTP-bound human MxA protein interacts with the nucleocapsids of Thogoto virus (Orthomyxoviridae).* J Biol Chem, 1999. **274**(7): p. 4370-6.
74. Kochs, G., et al., *Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 3153-8.
75. Pavlovic, J., O. Haller, and P. Staeheli, *Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle.* J Virol, 1992. **66**(4): p. 2564-9.
76. Zurcher, T., J. Pavlovic, and P. Staeheli, *Mechanism of human MxA protein action: variants with changed antiviral properties.* EMBO J, 1992. **11**(4): p. 1657-61.
77. Horisberger, M.A., *Interferon-induced human protein MxA is a GTPase which binds transiently to cellular proteins.* J Virol, 1992. **66**(8): p. 4705-9.
78. Engelhardt, O.G., et al., *Interferon-induced antiviral Mx1 GTPase is associated with components of the SUMO-1 system and promyelocytic leukemia protein nuclear bodies.* Exp Cell Res, 2001. **271**(2): p. 286-95.
79. Mayer, D., et al., *Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches.* J Proteome Res, 2007. **6**(2): p. 672-82.
80. Shen, H., et al., *Distinct activities of the DExD/H-box splicing factor hUAP56 facilitate stepwise assembly of the spliceosome.* Genes Dev, 2008. **22**(13): p. 1796-803.
81. Kota, K.P., et al., *Binding of ATP to UAP56 is necessary for mRNA export.* J Cell Sci, 2008. **121**(Pt 9): p. 1526-37.

82. Dufu, K., et al., *ATP is required for interactions between UAP56 and two conserved mRNA export proteins, Aly and CIP29, to assemble the TREX complex.* Genes Dev, 2010. **24**(18): p. 2043-53.
83. Yamazaki, T., et al., *The closely related RNA helicases, UAP56 and URH49, preferentially form distinct mRNA export machineries and coordinately regulate mitotic progression.* Mol Biol Cell, 2010. **21**(16): p. 2953-65.
84. Weber, F., et al., *Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses.* J Virol, 2006. **80**(10): p. 5059-64.
85. Di Paolo, C., et al., *Intramolecular backfolding of the carboxyl-terminal end of MxA protein is a prerequisite for its oligomerization.* J Biol Chem, 1999. **274**(45): p. 32071-8.
86. Meignin, C. and I. Davis, *UAP56 RNA helicase is required for axis specification and cytoplasmic mRNA localization in Drosophila.* Dev Biol, 2008. **315**(1): p. 89-98.
87. Ostertag, D., T.M. Hoblitzell-Ostertag, and J. Perrault, *Overproduction of double-stranded RNA in vesicular stomatitis virus-infected cells activates a constitutive cell-type-specific antiviral response.* J Virol, 2007. **81**(2): p. 503-13.
88. Janzen, C., G. Kochs, and O. Haller, *A monomeric GTPase-negative MxA mutant with antiviral activity.* J Virol, 2000. **74**(17): p. 8202-6.
89. Schumacher, B. and P. Staeheli, *Domains mediating intramolecular folding and oligomerization of MxA GTPase.* J Biol Chem, 1998. **273**(43): p. 28365-70.

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